Low-normal thyroid function and cardio-metabolic risk markers

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Colofon

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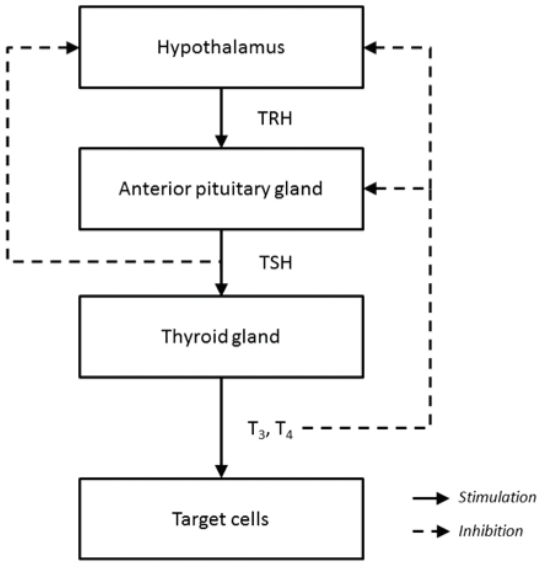
1.

General introduction and aims of this thesis

General introduction and aims of this thesis

The thyroid hormones triiodothyronine (T3) and thyroxine (T4) are synthesized by the follicular cells in the thyroid gland. Synthesis and secretion of thyroid hormones are regulated by thyroid stimulating hormone (TSH) which is produced by the thyrotroph cells in the anterior pituitary gland. In turn, TSH secretion is regulated by negative feedback of thyroid hormones and by stimulation of thyrotropin-releasing hormone (TRH), produced by the thalamus [figure 1]. Thyroid hormones have many physiological actions and essentially modulate all metabolic pathways [1]. T3 is commonly believed to be more biologically active as a regulator of metabolic processes [2,3]. The importance of thyroid hormones for development is underscored by observations showing that delayed diagnosis of congenital hypothyroidism, a condition of impaired thyroid hormone production, results in impaired brain development and cognitive impairment in humans, as confirmed in animal models [4,5]. TSH level is generally used to reflect the thyroid function status with respect to classification of subjects in euthyroidism (TSH within the reference range together with a free T4 (FT4) level within the reference range), (subclinical) hypothyroidism (elevated TSH together with a FT4 level which is within the reference range or decreased) and (subclinical) hyperthyroidism (suppressed TSH together with an FT4 and/or an free T3 (FT3) level which are within the reference range or being elevated) [6,7]. Consequently, a TSH level in the upper part of the reference range and/or a FT4 and FT3 level in the lower part of the reference range reflect a “low-normal” thyroid function status.

Figure 1: Thyroid hormone synthesis and regulation.



Thyroid hormones have effects on many metabolic pathways that affect atherosclerotic cardiovascular disease [8,9]. It is widely appreciated that overt hypothyroidism adversely effects cardiovascular morbidity and mortality [8-10]. Controversy remains to the risk of cardiovascular disease associated with subclinical hypothyroidism (SCH) [11-15]. Currently, it is unclear whether variations in thyroid function as inferred from plasma levels of TSH, FT4 and FT3 within the reference range impact on cardio-metabolic disorders.
As reviewed in chapter 2 there is accumulating evidence in support of the concept that low-normal thyroid function, i.e. higher TSH and/or lower free thyroid hormone levels within the euthyroid reference range, may play a pathogenic role in the development of several highly prevalent disorders such as atherosclerotic cardiovascular disease (CVD) [16-23]. Nonetheless, the extent to which low-normal thyroid function impacts on cardiovascular outcome is still unclear. Possible adverse effects of low-normal thyroid function on cardiovascular outcome such as stroke or coronary heart disease may be particularly relevant for specific populations, like younger people [22,23]. Alike subclinical hypothyroidism, low-normal thyroid function relates to a greater carotid artery intima media thickness (cIMT) and coronary artery calcification, which are established markers of (subclinical) atherosclerosis [18-21]. Low-normal thyroid function may also be associated with insulin resistance, obesity, the metabolic syndrome (MetS) and chronic kidney disease (CKD) [16,24-28]. Whereas the prevalence of non-alcoholic fatty liver disease (NAFLD), considered to represent the hepatic manifestation of MetS, is increased in (sub)clinical hypothyroidism [29]. Inconsistent effects of low-normal thyroid function on NAFLD have been reported so far [30-32].

Low-normal thyroid function and cardio-metabolic risk markers

The mechanisms responsible for the association of (subclinical) atherosclerosis with low-normal thyroid function are still incompletely understood. Low-normal thyroid function may give rise to modest increases in plasma levels of total cholesterol and apolipoprotein B (apoB)-containing lipoproteins, such as very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and low density lipoprotein (LDL) [28,33,34]. Subendothelial retention of apoB-containing lipoproteins is a well-known process, which takes place early in the process of atherosclerosis [35,36]. Retained lipoproteins in the arterial wall subsequently provoke an inflammatory response by stimulating local synthesis of proteoglycans involved in inflammatory processes which accelerate further lipoprotein retention [36]. Furthermore, low-normal thyroid function may convey changes in high density lipoprotein (HDL) function, which conceivable contribute to impaired oxidative stress defense [37]. In this regard, it is important that HDL contain paraoxonase-1 (PON-1) which has anti-oxidative and probably also anti-inflammatory activity [38,39]. Thyroid function status may also affect pro- and anti-inflammatory biomarkers, including adipokines and tumor necrosis factor alfa (TNF-α) [40-43].

Aim of the thesis

The aim of this thesis is to investigate the relationship of low-normal thyroid function with novel lipid and non-lipid biomarkers which have been recently identified to be involved in the pathogenesis of atherosclerotic CVD. Furthermore, the relationship of non-alcoholic fatty liver disease (NAFLD) with thyroid function status will be studied.

Outline of the thesis

In **chapter 2** we review the relationship of low-normal thyroid function with CVD, plasma lipids and lipoprotein function. Furthermore the relationship of low-normal thyroid function with MetS, CKD and NAFLD, and the responsible mechanisms are discussed. This review has been published in 2015 and covers the literature until that time.

Chapter 3 describes a cross-sectional study of Type 2 diabetes mellitus (T2DM) and non-diabetic subjects. In SCH the secretion of large VLDL particles by the liver is increased, whereas plasma triglyceride clearance is likely to be unaltered. In this chapter, we have tested the hypothesis that low-normal thyroid function confers altered lipoprotein subfraction levels. We also have investigated whether such possible relationships are modified in T2DM.

In **chapter 4** the relationship of plasma apolipoprotein (apo) E with low-normal thyroid function is determined in euthyroid subjects with and without T2DM. ApoE plays an important role in the metabolism of triglyceride-rich apoB-containing lipoproteins. ApoE is also important for hepatic VLDL production. In addition, VLDL-associated apoE contributes to impaired clearance of these lipoproteins. The total plasma apoE concentration strongly correlates with triglycerides and is elevated in subjects with MetS. Plasma apoE has been documented to be associated positively with CVD. Given the prominent role of increased hepatic VLDL production in diabetic dyslipidemia our study is again focused on subjects with and without T2DM.

In **chapter 5** we cross-sectionally studied the relationships of plasma pre β-HDL with thyroid function in euthyroid subjects with and without T2DM. Pre β-HDL particles are small lipid poor HDLs which act as initial acceptors of cell-derived cholesterol. Pre β-HDL particles play an important role in the reverse cholesterol transport pathway, whereby cholesterol is transported from peripheral cells back to the liver for biliary transport and excretion in the feces. Remarkably, higher plasma pre β-HDL concentrations have been observed in subjects with cardiovascular disease. Higher plasma pre β-HDL levels may associate with a greater cIMT. We hypothesized that variation in FT4 within the euthyroid range may effect HDL metabolism by affecting plasma pre β-HDL. We also investigate whether such relationships are modified in T2DM.

Chapter 6 discusses a population-based study of euthyroid subjects recruited form the general population (the PREVEND (Prevention of Renal and Vascular END-Stage Disease) cohort). The PREVEND study is a prospective study in which the role of elevated urinary albumin excretion as well as lipid and non-lipid markers in progression of renal and cardiovascular disease is determined. In these subjects we have investigated the association of serum paraoxonase-1 (PON-1) with thyroid function parameters.

Chapter 7 concerns the relationship of low-normal thyroid function with TNF-α. TNF-α is an established mediator of apoptosis, inflammation and the innate immune system response to different forms of stress, like ischemia. TNF-α seems to be important in the development of coronary heart disease and plaque formation. Moreover, plasma TNF-α may correlate positively with cIMT. Higher TNF-α levels are found in humans with SCH. T2DM is characterized by low-grade inflammation. Therefore, this cross sectional study was initiated to determine the relationship of low-normal thyroid function with TNF-α in euthyroid subjects with and without T2DM.

Chapter 8 describes a cross-sectional study carried out among euthyroid subjects with and without MetS. In this study we have investigated the possible relationships of plasma leptin, adiponectin and the leptin/adiponectin (L/A) ratio with TSH an free T4. Thyroid function status is likely to affect circulating levels of leptin and adiponectin, adipokines, which exert pro-and anti-inflammatory properties, respectively. Leptin has been reported to decrease and adiponectin to increase after levothyroxine supplementation in SCH, which provide our rationale to hypothesize that plasma leptin/adiponectin (L/A) ratio may be higher in subjects with low-normal thyroid function.

Finally, **chapter 9** describes a population-based cohort study of participants living in the North of the Netherlands (Lifelines Cohort Study). Because the liver plays a crucial role in the metabolism of cholesterol and triglycerides, and thyroid hormones affect hepatic lipid homeostasis, we aimed to determine the relationship of NAFLD with TSH, FT4 and FT3 in euthyroid subjects.

Summary, general discussion and future outlook
In **chapter 10** the main results, described in chapter 2-9, are discussed.

Chapter 11 contains a summary in Dutch.

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2.

Low-normal thyroid function and the pathogenesis of common cardio-metabolic disorders

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Eur J Clin Invest 2015;45:494-503

Abstract

Background: Subclinical hypothyroidism may adversely affect the development of cardiovascular disease (CVD). Less is known about the role of low-normal thyroid function, i.e. higher thyroid-stimulating hormone and/or lower free thyroxine levels within the euthyroid reference range, in the development of cardio-metabolic disorders. This review is focused on the relationship of low-normal thyroid function with CVD, plasma lipids and lipoprotein function, as well as with metabolic syndrome (MetS), chronic kidney disease (CKD) and non-alcoholic fatty liver disease (NAFLD).

Materials and methods: This narrative review, which includes results from previously published systematic reviews and meta-analyses, is based on clinical and basic research papers, obtained via MEDLINE and Pubmed up to November 2014.

Results: Low-normal thyroid function could adversely affect the development of (subclinical) atherosclerotic manifestations. It is likely that low-normal thyroid function relates to modest increases in plasma total cholesterol, LDL cholesterol and triglycerides, and may convey pro-atherogenic changes in lipoprotein metabolism and in HDL function. Most available data support the concept that low-normal thyroid function is associated with MetS, insulin resistance and CKD, but not with high blood pressure. Inconsistent effects of low-normal thyroid function on NAFLD have been reported so far.

Conclusions: Observational studies suggest that low-normal thyroid function may be implicated in the pathogenesis of CVD. Low-normal thyroid function could also play a role in the development of MetS, insulin resistance and CKD, but the relationship with NAFLD is uncertain. The extent to which low-normal thyroid function prospectively predicts cardio-metabolic disorders has been insufficiently established so far.

Introduction

The high prevalence of thyroid dysfunction in the population has considerable consequences for a number of health issues, including cardiovascular disease (CVD), metabolic syndrome (MetS), chronic kidney disease (CKD) and non-alcohol fatty liver disease (NAFLD) [1]. Overt hypothyroidism adversely affects cardiovascular morbidity and mortality [2]. Subclinical hypothyroidism (SCH), commonly defined as a plasma thyroid-stimulating hormone (TSH) level above the reference range (above 4 to 4.5 mU/L), together with a plasma free thyroxine (FT4) level within the reference range, is a well-recognized entity [3]. SCH is common with a prevalence between 4.6 and 8.5% in adults, which rises to 15% in the elderly [4,5]. More recently, the concept is emerging that low-normal thyroid function, i.e. higher TSH and/or lower FT4 levels within the euthyroid reference range, even when determined at a single time-point [6], could have a negative impact on cardio-metabolic disorders [1,7].

This review is focused on the relationship of low-normal thyroid function with CVD, lipids and lipoprotein function, MetS, CKD and NAFLD, and the responsible mechanisms thereof.

Methods of data collection

This narrative review is based on clinical and basic research papers, including systematic reviews and meta-analyses, that were identified using MEDLINE and Pubmed databases up to November 2014. Key search terms were thyroid function, low-normal thyroid function, TSH or FT4 in combination with cardiovascular disease, intima media thickness, cholesterol metabolism, lipid metabolism, plasma lipoproteins, metabolic syndrome, obesity, kidney function, chronic kidney disease or non-alcoholic fatty liver disease. Only studies published in English language were considered.

Subclinical hypothyroidism, low-normal thyroid function and atherosclerotic cardiovascular disease

The possible impact of SCH on CVD has received considerable attention. Three meta-analyses, that were based on population-based studies, have assessed the strength of the association between SHC and CVD [8-10] (Table 1). Ochs et al. reported that SCH is associated with a non-significant 20 % (10 studies; 95 % confidence interval (CI), -3-49 %) higher relative risk for coronary heart disease (CHD) in a meta-analysis involving a total of 14449 participants (14021 subjects were included in the SCH-CHD analysis) [8]. The risk of cardiovascular mortality appeared to be greater in those studies in which mean age of participants was < 65 years compared to studies in which mean age was ≥ 65 years (relative risk, 1.51 (95 % CI, 1.09-2.09) vs. 1.20 (95 % CI, 0.90-1.22) [8]. Razvi et al. observed a modestly increased prevalence of ischemic heart disease (IHD) attributable

to SCH (12 studies; odds ratio, 1.23 (95 % CI, 1.02-1.48); 27267 subjects) [9]. The effect of SCH on incident IHD was not significant (5 studies; odds ratio, 1.27 (95 % CI, 0.95-1.69); 9627 subjects) [9]. In line with the first meta-analysis, they reported an increased prevalence and incidence of ischemic heart disease in conjunction with SCH in studies including subjects < 65 years of age (odds ratio, 1.57 (95 % CI, 1.19-2.06) and 1.68 (95 % CI, 1.27-2.23), respectively), but not in studies which only included subjects ≥ 65 years of age (odds ratios, 1.01 (95% CI, 0.87-1.18) and 1.02 (95 % CI, 0.85-1.22), respectively) [9]. A similar age-dependent trend was found for cardiovascular mortality [9]. A third meta-analysis by Singh et al. documented that SCH confers an increased risk of both prevalent CHD (5 studies; relative risk, 1.53 (95 % CI, 1.31-1.79); 11495 subjects), and incident CHD (3 studies; relative risk, 1.19 (95 % CI, 1.02-1.38); 8076 subjects) [10]. The risk of cardiovascular death was also increased in SCH subjects (relative risk, 1.28 (95 % CI, 1.02-1.60)), although the association of SCH with all-cause mortality was not significant (relative risk, 1.15 (95 % CI, 0.99-1.26)) [10].

Table 1. Summary of results of 3 meta-analyses on the association of subclinical hypothyroidism (SCH) with cardiovascular disease.

Reference	Number of studies included	Number of participants	Outcome/ follow-up	Relative risk (95 % confidence intervals)
Ochs; Ref. 8 (2008)	10	SCH: 1491 euthyroid: 12530	incident coronary heart disease follow-up: 3.7 to 20 years	1.20 (0.97-1.49)
Razvi; Ref. 9 (2008)	12	SCH: 2399 euthyroid: 24868	prevalent ischemic heart disease	1.23 (1.02-1.48)
Razvi; Ref. 9 (2008)	5	SCH: 954 euthyroid: 8673	incident ischemic heart disease follow-up: median 8.6 years	1.27 (0.95-1.69)
Singh; Ref. 10 (2008)	5	SCH: ns euthyroid: ns	prevalent coronary heart disease	1.53 (1.31-1.79)
Singh; Ref. 10 (2008)	3	SCH: ns euthyroid: ns	incident coronary heart disease follow-up: 4, 12 and 20 years	1.19 (1.02-1.38)

ns: not specified

Carotid intima–media thickness (cIMT) is a predictor of CHD and stroke, and represents an established surrogate marker of subclinical atherosclerosis [11]. cIMT is probably greater in overt hypothyroidism compared to euthyroidism, and was found to decrease after levothyroxine replacement [12]. A German study including subjects across the range from hypothyroidism to hyperthyroidism showed that cIMT was smaller in SCH, as indicated by TSH levels above the reference range [13]. However, mean age was lower and there was a higher frequency of women in the elevated SCH group, which could have confounded the smaller cIMT in the SCH group. Furthermore, there was no significant linear relationship of cIMT with the TSH level [13]. In another study, no significant inverse relationship of cIMT with TSH was found after adjustment for age, sex and cardiovascular risk factors [14]. Of note, a meta-analysis of observational studies comprising 3602 participants, demonstrated that SCH is associated with a greater cIMT, particularly at TSH > 10 mU/L [15]. This meta-analysis lends support to the hypothesis that SCH may confer increased risk of subclinical atherosclerosis.

The Hunt Study prospectively examined the association of CHD mortality with TSH levels within the reference range in a Norwegian cohort of 25313 men and women without known thyroid disease, CVD or diabetes mellitus at baseline [16]. In euthyroid women, mortality from CHD was positively and independently associated with TSH within the reference range (hazard ratio middle vs. lower tertile: 1.41 (95 % CI, 1.06-1.87); upper vs. lower tertile: 1.45 (95 % CI, 1.01-2.08-) [16]. In non-smoking men, there was non-significant association of CHD mortality with high-normal TSH. These important findings suggest that low-normal thyroid function may increase CVD risk [16]. On the other hand, a prospective study from the UK among 1191 individuals did not show a significant relationship between (cardiovascular) mortality and variation in TSH levels within the euthyroid reference range, but this survey was primarily focused on subclinical hyperthyroidism [17]. In a cross-sectional study including Caucasian non-smoking, middle-aged euthyroid subjects, cIMT was inversely and independently related to FT4 [18]. Likewise, cIMT was associated inversely with FT4 among euthyroid Japanese subjects [19]. Although these results agree with the hypothesis that low-normal thyroid function could play a role in the development of atherosclerosis, large-scale prospective studies both with incident cardiovascular events and with cIMT changes over time as outcome are required to more definitely test whether variations in thyroid function within the normal range indeed confer increased CVD risk.

Changes in plasma lipoprotein levels consequent to subclinical hypothyroidism and low-normal thyroid function

SCH results in modest increases in plasma total cholesterol, LDL cholesterol and triglycerides [20,21]. (Table 2). The lipoprotein abnormalities in SCH are essentially normalized after levothyroxine treatment (Table 2). Minor and inconsistent changes in HDL cholesterol have been reported in SCH [21]. Plasma lipoprotein (a) (Lp(a)), a pro-atherogenic

subfraction of LDL that is formed by disulfide bridges between apolipoprotein (apo)B and apo(a), is probably unchanged in SHC [21], although Lp(a) is markedly increased in overt hypothyroidism [22].

We retrieved 9 studies comprising > 500 subjects (90041 individuals), which evaluated the effect of low-normal thyroid function on plasma (apo)lipoproteins [16,19,23-29] (Table 3). A positive relationship of plasma total cholesterol, LDL cholesterol and triglycerides with TSH was found in 3, 1 and 3 of these reports, respectively. Four studies did not show a relationship of plasma total cholesterol and LDL cholesterol with TSH, whereas in 2 studies the relationship with triglycerides was not significant. The relationship of these lipoprotein measures with FT4 was assessed in 4 studies. Plasma total cholesterol and LDL cholesterol were positively related to FT4 in one study, but inversely in another. Higher plasma triglycerides were related to lower FT4 levels in two studies and to higher FT4 in one paper. Variable effects of low-normal thyroid function on HDL cholesterol were reported. The relationships of lipoprotein measures with TSH and/or FT4 were modest (correlation coefficients ≤0.12). Collectively, these studies suggests that low-normal thyroid function may give rise to small increases in plasma apoB-containing lipoproteins, in keeping with such changes in lipoprotein levels in SCH.

Table 2. Effects of overt and subclinical hypothyroidism on plasma (apo)lipoproteins, and of levothyroxine treatment in subclinical hypothyroidism.

	Overt hypothyroidism	**Subclinical hypothyroidism**	**Levothyroxine treatment**
Total cholesterol	↑	↑, ns	↓, ns
LDL cholesterol	↑	↑, ns	↓, ns
HDL cholesterol	↑	↓, ns	↑,ns
Triglycerides	↑	↑, ns	↓, ns
Apolipoprotein B	↑	↑	↓
Apolipoprotein A-I	↑	ns	ns
Lp(a)	↑	ns	ns

HDL: high density lipoproteins; LDL: low density lipoproteins; Lp(a): lipoprotein (a).
↑: increased; ↓: decreased; ns: no significant effect.

Table 3. Relationships of plasma (apo)lipoproteins with thyroid function parameters in euthyroid subjects as determined by cross-sectional analyses in population-based studies (all studies included > 500 individuals).

Reference	Number of subjects; Analysis	Total cholesterol	LDL cholesterol	HDL cholesterol	Triglycerides	apoB	apoA-I
Ref. 16 (2007)	n= 27727 adjusted for age, smoking and prandial state men and women separately	TSH: +	TSH: +	TSH: -	TSH: +		
Ref. 23 (2007)	n= 1581 crude men and women combined	TSH: ns FT4: - FT3: -	TSH: ns FT4: - FT3: -	TSH: + FT4: ns FT3: ns	TSH: + FT4: - FT3: -	TSH: ns FT4: ns FT3:-	TSH: + FT4: ns FT3: ns
Ref. 19 (2009)	n=643 crude men and women combined	TSH: ns FT4: ns	TSH: ns FT4: ns	TSH: - FT4: ns	TSH: ns FT4: +		
Ref. 24 (2009)	n-44196 crude men and women separately	FT4:+	FT4:+	FT4:+	FT4:-		
Ref. 25 (2009)	n=949 crude postmenopausal women	TSH:+	TSH:+	TSH: ns	TSH:+		
Ref. 26 (2010)	n=2771 adjusted for age, sex and BMI	TSH: + FT4: ns	TSH: ns FT4: ns	TSH: ns FT4: +	TSH: + FT4: ns		
Ref. 27 (2011)	n=7720 adjusted for age, sex, BMI, season, menopausal status	TSH: +	TSH: +	TSH: ns	TSH: +		
Ref. 28 (2011)	n=1240 crude	TSH: ns	TSH: ns	TSH: ns	TSH: ns		
Ref. 29 (2012)	n=3364 crude men and women combined	TSH: ns	TSH: ns	TSH: +	TSH: ns		

MI: body mass index; FT4: free thyroxine; FT3: free triiodothyronine; TSH: thyroid-stimulating hormone; n: number; ns: not significant. Positive (+) and negative (-) associations are indicated.

Effects of thyroid hormones on lipid homeostasis, lipoprotein metabolism and lipoprotein function

Thyroid hormones induce the expression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key regulator of cholesterol synthesis [21]. The LDL receptor gene and one of its main regulatory factors, sterol regulatory element-binding protein-2 (SREBP-2), contain a thyroid hormone responsive element [30]. Consequently, LDL receptor expression is under dual control of thyroid hormones. LDL clearance is increased by the action of thyroid hormones. This results in lower plasma LDL cholesterol in hyperthyroidism and higher levels in hypothyroidism, despite stimulating effects of thyroid hormones on hepatic cholesterol synthesis [21]. More recently, it has been shown that proprotein converstase subtilisn-kexin type 9 (PCSK9) is intricately implicated in LDL metabolism [31]. PCSK9 is a secreted protease that binds to the extracellular domain of the LDL receptor, thereby targeting it for lysosomal degradation after endocytosis. PCSK9 prevents LDL receptor recycling to the cell surface and decreases LDL receptor abundancy. Plasma PCSK9 levels are probably physiologically relevant, because LDL clearance is decreased at higher PCSK9 plasma levels [32]. PCSK9 expression is also regulated by SREBP-2 [31]. Low-normal thyroid function, as reflected by high-normal TSH levels, may confer increased plasma PCSK9 levels in non-obese individuals, suggesting that thyroid function status may affect cellular cholesterol trafficking by affecting LDL receptor expression via PCSK9 regulation [33]. Of further note, there is some evidence to suggest that TSH could have a direct effect on HMG-CoA expression [34]. Finally, biliary excretion of cholesterol and neutral steroids is decreased, whereas intestinal cholesterol absorption is increased in hypothyroidism [35].

Thyroid hormones increase the mobilization of stored triglycerides by stimulating adipose tissue lipolysis [21,36]. Circulating free fatty acid and glycerol levels are increased in hyperthyroidism, which enhances delivery of free fatty acids to the liver for subsequent re-esterification to triglycerides [36]. Thyroid hormones stimulate hepatic fatty acid β-oxidation as well [36]. As a result of these divergent actions, hypothyroidism most likely promotes hepatic triglyceride accumulation [7,36], which represents a main driving force for increased production of large very low density lipoprotein (VLDL) particles as evidenced in metabolic syndrome (MetS), and Type 2 diabetes mellitus (T2DM) [37,38]. Furthermore, VLDL particle clearance is decreased in hypothyroidism which is due to impaired activity of lipoprotein lipase [21] and probably also to impaired hepatic VLDL removal via LDL receptor-related protein 1 [39]. Low-normal FT4 levels may predict an increased concentration of large VLDL particles and a greater VLDL size [40], raising the possibility that abnormalities in triglyceride metabolism may represent an early abnormality in the setting of low-normal thyroid function.

Thyroid hormones are also involved in HDL metabolism by affecting the regulation of a number of protein factors such as lecithin:cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP) and hepatic lipase [21,41-43]. The plasma activities

of these proteins are increased by thyroid hormones [41-43]. The HDL-associated enzyme, LCAT, esterifies free cholesterol to cholesteryl esters, thereby promoting the conversion of lipid-poor pre β-HDL to larger, spherical HDL particles [44]. Subsequently, HDL-derived cholesteryl esters are transferred to triglyceride-rich lipoproteins by the action of CETP. As a consequence of this cholesteryl ester transfer (CET) process, the cholesterol content of HDL is decreased [44]. At the same time, triglycerides are transferred in the opposite direction to HDL, resulting in triglyceride-enriched HDL particles. HDL triglycerides are then hydrolyzed by hepatic lipase, giving rise to smaller-sized HDL particles [44]. By a comparable mechanism, the CET process also contributes to the generation of atherogenic small-dense LDL. The CET process thus contributes to an unfavorable plasma lipoprotein profile, with possible consequences for atherosclerosis development [45,46,47]. Alterations in LCAT, CETP and hepatic lipase act in concert to increase HDL cholesterol and HDL size in severe hypothyroidism, with opposite HDL changes in hyperthyroidism [21,43]. Plasma CET was found to be related to high-normal TSH in euthyroid T2DM subjects but not in euthyroid non-diabetic individuals [48]. In this report, the positive relationship of plasma CET with triglycerides in T2DM was more outspoken with higher TSH levels [48]. These data would underscore the concept that low-normal thyroid function may adversely affect a pro-atherogenic lipid biomarker in conjunction with chronic hyperglycemia and hypertriglyceridemia.

The importance of HDL functional properties beyond HDL cholesterol levels is increasingly appreciated [49]. Besides other athero-protective functions, HDL inhibits LDL from oxidative modification, thereby protecting against oxidative stress [49]. Importantly, LDL oxidizability is increased in overt hypothyroidism [50,51]. Oxidative stress markers are elevated in SCH [52,53], whereas high-normal TSH levels within the euthyroid range associate with elevations in oxidized LDL [54]. In agreement with the hypothesis that low-normal thyroid function may negatively impact on in the ability of HDL to protect LDL from oxidative modification, impaired HDL anti-oxidative capacity was determined by low-normal FT4 levels in T2DM [55], a condition characterized by enhanced oxidative stress [56].

Low-normal thyroid function and the metabolic syndrome

In a cross-sectional analysis involving 2703 non-diabetic participants of the PREVEND (Prevention of Renal and Vascular End stage Disease) study (www.PREVEND.org; Groningen, the Netherlands), low-normal thyroid function, in particular a low FT4 level within the euthyroid reference range was associated with 4 of the 5 MetS components (waist circumference, triglycerides, HDL cholesterol triglycerides and glucose), but not significantly with blood pressure [23]. A high-normal TSH level within the euthyroid reference range was also associated with an increased prevalence of MetS in the Healthy ABC study, but did not predict new-onset MetS during follow-up [57]. The presence of MetS

was associated with low-normal FT4 levels in Korean people [24], and post-menopausal women [25]. Accordingly, more severe insulin resistance (homeostasis model assessment) was associated low-normal FT4 levels in the PREVEND cohort [23] and in a Mexican survey [26]. On the other hand, SCH did neither predict increased prevalence of MetS, nor of its individual components in the Mexican study [26].

The complex interactions of thyroid function with obesity has been reviewed elsewhere [58]. Thyroid hormones increase resting energy expenditure [59]. Although the increase in body weight in overt hypothyroidism seems to be mainly due to fluid retention [58], small differences in thyroid function even within the normal range are probably relevant for adiposity, as judged by a positive association of BMI with TSH and an inverse association with FT4, as well as by a higher prevalence of obesity with higher TSH levels [60]. A positive association of BMI and waist circumference with TSH was also observed in adult men and women participating in the National Health and Nutrition Examination Survey [61]. In obese children, on the other hand, both TSH and FT4 levels were higher compared to non-obese children [62]. This suggests that the hypothalamic-pituitary-thyroid axis may be activated in early-onset obesity, which could in part be attributed to higher leptin levels [58].

Hypothyroidism leads to insulin resistance in striated muscle and adipose tissue, which is ascribed to decreased translocation of GLUT4 to the cell membrane, thereby impairing glucose transport [63]. Additionally, insulin clearance may be diminished in hypothyroidism coinciding with higher levels of counter-regulatory hormones [64]. Thus, despite increased gluconeogenesis in hyperthyroidism, and enhanced glucose-stimulated insulin secretion in overt hypothyroidism and SCH, plasma glucose levels tend to be higher in hypothyroidism [65,66]. In line, a low-normal FT4 may relate to somewhat higher fasting plasma glucose [23].

A meta-analysis comprising 6 cross-sectional studies documented that SCH confers a small increase in systolic blood pressure of 1.89 (95 % CI, 0.98-2.80) mm Hg and in diastolic blood pressure of 0.75 (95 % CI, 0.24-1.27) mm Hg [67]. In agreement, effects low-normal thyroid function on systemic blood pressure are probably minimal. Both in the Busselton Health study and in the PREVEND study, blood pressure was not significantly associated with low-normal thyroid function [23,68]. Likewise, neither systolic nor diastolic blood pressure was correlated with TSH or FT4 in euthyroid Japanese subjects [19]. In other surveys, positive associations of systolic or diastolic blood pressure with TSH were found [25,26,69], although positive associations of blood pressure with FT4 levels have also been reported [24].

Hypothyroidism, low-normal thyroid function and glomerular filtration rate

Overt thyroid dysfunction exerts major functional effects on the kidney, which include changes in renal blood flow, glomerular filtration rate, tubular function, as well as in sodium and water balance [70,71]. Estimated glomerular filtration rate (eGFR) was found to increase from 70 to 83 ml/min/1.73 m^2 after thyroid hormone treatment for overt hypothyroidism, and to decrease from 135 to 96 ml/min/1.73 m^2 after thyreostatic drug treatment for hyperthyroidism [72]. Accordingly, there was a strong correlation ($r^2 = 0.69$) between the changes in FT4 after treatment of hypo- or hyperthyroidism and the changes in eGFR [71]. In line, in patients with stage 2 to 4 chronic kidney disease (CKD; eGFR between 15 and 90 ml/min/1.73 m^2), the rate of decline in eGFR over time is attenuated after thyroid hormone replacement treatment in SCH [73].

CKD ≥ stage 3 (eGFR < 60 ml/min/1.73 m^2), is estimated to afflict more than 8 million US inhabitants [74]. Among 461,607 veterans (less than 5 % women) with CKD ≥ stage 3 (only 0.4 % of subjects with end-stage renal failure) , the multivariably adjusted risk of concurrent hypothyroidism (defined as TSH > 5 mU/L or thyroid hormone replacement) was found to be 18 % higher for every 10 mL/min/1.73m^2 lower eGFR [75]. Such an association of CKD with hypothyroidism was observed after controlling for a number of sociodemographic variables, including age, sex, race/ethnicity, concomitant CVD, malignancy and other illness states [75]. This is important, because comorbidities associated with CKD in the absence of primary thyroid diseases may confound the interpretation of the relationship of CKD with thyroid functional status. End-stage renal failure may give rise to diminished pituitary TSH secretion, consistent with the non-thyroidal illness syndrome, which hampers the interpretation of thyroid functional status in severe CKD [76]. In a cross-sectional population-based study among 26619 Norvegian individuals with a TSH level within the euthyroid reference range, eGFR was inversely associated with TSH [77]. In that survey, eGFR was on average 2.7 mL/min/1.73m^2 lower at the highest vs. the lowest TSH tertile within the euthyroid reference range (eGFR 80.3 vs. 83.0 mL/min/1.73m^2), taking account of age, sex and smoking [77]. Subjects with a TSH level in the upper tertile of the euthyroid reference range had a 31 % higher risk of CKD ≥ stage 3 vs. subjects with a TSH levels in the lowest tertile [77]. In view of these results, low-normal thyroid function should probably be regarded as a risk determinant of CKD.

Importantly, in a meta-analysis involving more than a million individuals, eGFR< 60 ml/min/1.73 m^2 predicted all-cause and cardiovascular mortality independent from conventional risk factors, and independent from but multiplicatively associated with albuminuria [78]. For these reasons, CKD is considered to be a CHD risk equivalent [74]. Interestingly, a robust association between NAFLD and CKD progression has been reported recently [79]. Therefore, it is tempting to speculate that low-normal thyroid function could play a pathogenic role in the interplay between CKD, NAFLD and CVD development.

Hypothyroidism, low-normal thyroid function and non-alcohol fatty liver disease

NAFLD includes a broad spectrum of pathology ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis [80,81]. NAFLD also predisposes to hepatocellular carcinoma. NAFLD has become a become a leading cause of liver disease worldwide, and it is estimated that NAFLD afflicts more than 30% of American adults [82]. NAFLD is considered to reflect the hepatic component of MetS, given the strong association with insulin resistance, hypertension, obesity and dyslipidemia [83]. Accumulating evidence supports an association between NAFLD and increased risk of CVD [84,85], which at least in part independent from an association with CKD [80].

Thyroid hormones play a important and complex role in the hepatic lipid metabolism (see previous section). Thyroid hormones do not only increased hepatic lipogenesis, but also enhance fatty acid β-oxidation [86]. Agonists of thyroid hormone receptor β, i.e. the subunit which is naturally expressed in hepatocytes, diminish hepatic fat accumulation in animal studies [87]. Although increased fatty acid β-oxidation is anticipated to attenuate hepatic fat accumulation, this process may at the same time result in excessive production of reactive oxygen species by mitochondria, which is anticipated to induce hepatocyte damage. Thyroid hormones could also influence hepatic fat accumulation, and the subsequent development of fibrosis via an effect on adiponectin regulation, an adipokine which has the ability to stimulate fatty acid oxidation and to inhibit *de novo* lipogenesis [36,88,89].

Subjects with hypothyroidism are about 1.5 to 2 times more likely to have biopsy-proven or ultrasonography confirmed NAFLD [90,91]. NAFLD is associated with hypothyroidism in a dose-dependent manner, independent of metabolic risk factors (SCH: odds ratio,1.36 (95 % CI, 1.16-1.61); overt hypothyroidism: odds ratio 1.71 (95 % CI, 1.10-2.66) [92]. Likewise, elevations in serum alanine aminotransferase (ALT), a proposed surrogate marker of NAFLD [85,92], are associated with a higher TSH level across the spectrum of hypo- to hyperthyroidism [93]. A recent systematic review, including 11 studies, suggested that NAFLD is related to hypothyroidism, although this association has not uniformly been reported [36].

There are a only few studies which investigated the association of NAFLD with variations in thyroid function within the euthyroid range. Among 878 elderly Chinese subjects, NAFLD (prevalence 25.9 %, determined by ultrasonography) was independently associated with lower FT4 levels [94]. Likewise, NAFLD (prevalence 26.5 %, determined by ultrasonography) was associated with higher TSH and lower FT4 levels in another study in 739 Chinese subjects [95]. In a German study (3661 individuals), an association of NAFLD (based on ultrasonorography and ALT elevations) with lower FT4, but not with higher TSH levels was documented [96]. In a community-based Chinese survey study among euthyroid 1322 adults, TSH levels were higher in female subjects with NAFLD, but tis difference

disappeared after adjustment for adiposity [97]. In euthyroid subjects with biopsy-proven NAFLD, NASH was found to be positively associated with high-normal TSH levels within the euthyroid range [98]. On the other hand, NAFLD, determined by ultrasonography, was more prevalent in subjects with low TSH level among 832 Iranian subjects, most of them being euthyroid [99]. Furthermore, in a study comprising 82 Caucasian euthyroid subjects with and without MetS, a high-normal TSH level, was suggested to attenuate ALT elevations in the context of MetS and insulin resistance [89]. Thus, it is still unclear whether low-normal thyroid function within the euthyroid range relates to NAFLD. Methodological issues with respect to the assessment of NAFLD, as well as ethnic differences in susceptibility for liver fat accumulation could in part explain the discrepancies.

Conclusions

Evidence from observational studies is accumulating which supports the concept that low-normal thyroid function, i.e. higher TSH and/or lower FT4 levels within the euthyroid reference range, could adversely affect (subclinical) atherosclerosis. It is likely that low-normal thyroid function confers modest increases in plasma total cholesterol, LDL cholesterol and triglycerides, and may convey pro-atherogenic changes in lipoprotein-mediated processes and in HDL function, which conceivably contribute to impaired oxidative stress defense. Low-normal thyroid function may play a pathogenetic role in the development of MetS, obesity, insulin resistance and CKD, but effects on blood pressure are minimal. NAFLD is likely to be associated with SCH, but inconsistent effects of low-normal thyroid function on NAFLD have been reported so far. As yet, little information is available with respect to the extent to which low-normal thyroid function prospectively predicts the development of cardio-metabolic disorders. This overview provides a rationale to prospectively test the effect of thyroid hormone supplementation in subjects with low-normal thyroid function on (biomarkers of) cardiometabolic disorders.

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3.

Low normal thyroid function as a determinant of increased large very low density lipoprotein particles

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Abstract

Objectives: Low-normal thyroid function may relate to increases in plasma cholesterol and triglycerides, but effects on lipoprotein subfractions are largely unknown. Associations of alterations in lipoprotein metabolism and functionality with low-normal thyroid function could be more pronounced in Type 2 diabetes mellitus (T2DM). We determined relationships of plasma lipids and lipoprotein subfractions with thyroid stimulating hormone (TSH) and free thyroxine (free T_4) in euthyroid subjects, and assessed whether such relationships are modified in the context of T2DM.

Design and Methods: TSH, free T_4, (apo)lipoproteins and lipoprotein subfractions (nuclear magnetic resonance spectroscopy) were measured after an overnight fast in 61 T2DM subjects and 52 non-diabetic subjects.

Results: TSH and free T_4 were similar in T2DM and non-diabetic subjects. Plasma triglycerides, large very low density (VLDL) particles, VLDL size and small low density lipoprotein (LDL) particles were increased, whereas high density lipoprotein (HDL) cholesterol was decreased in T2DM subjects ($p \leq 0.05$ for each). Age-, sex-, and diabetes status-adjusted multivariable linear regression analysis demonstrated that plasma triglycerides were associated positively with TSH (β=0.196, p=0.039). Large VLDL particles (β=-0.215, p=0.020) and VLDL size were inversely associated with with free T_4 (β=-0.285, p<0.001). These relationships were not significantly modified by diabetes status (interaction terms: p>0.10 for each). In all subjects combined, LDL and HDL subfraction characteristics were not significantly related to thyroid function status.

Conclusions: Low-normal thyroid function may confer increased plasma triglycerides, large VLDL particles and increased VLDL particle size. These relationships are not to a major extent modified in the context of T2DM.

Highlights

- TSH, free T_4 and lipoprotein subfractions (NMR) were measured in euthyroid subjects
- 61 Type 2 diabetic and 52 non-diabetic subjects were studied in the fasting state
- Low-normal thyroid function was associated with large VLDL in all subjects combined
- Low-normal thyroid function may adversely affect triglyceride metabolism

Introduction

The high prevalence of thyroid function abnormalities in the general population provides a rationale to determine the consequences of mild thyroid dysfunction for a number of health issues including cardio-metabolic disorders [1-4]. Each person probably has a rather narrow individual set-point of thyroid function status [5]. It is, therefore, likely that single measurements of circulating thyroid-stimulating hormone (TSH) and thyroid hormones provide relevant information regarding the relationship of thyroid function with cardiovascular and metabolic biomarkers [3].

It is important that low-normal thyroid function, as reflected by higher TSH and/or lower thyroid hormone levels within the euthyroid range, probably confers higher plasma total cholesterol, triglycerides and apolipoprotein B (apoB) concentrations [6-10]. The concept is also emerging that low-normal thyroid function could adversely affect atherosclerosis susceptibility [11-13], although this issue has not been unequivocally settled at present.

In subclinical hypothyroidism the secretion of large very low density lipoprotein (VLDL) particles by the liver has been reported to be increased [14], whereas plasma triglyceride clearance is likely to be unaltered [14,15]. Little is currently known about the effect of low-normal thyroid function on lipoprotein subfraction levels. All major lipoprotein fractions are highly heterogeneous in size, structure and composition, which may have implications of their measurement for improved prediction of cardiometabolic disorders [16-18]. We have recently observed that the putative effects low-normal thyroid function on several cardio-metabolic biomarkers, i.e. an increase in the plasma cholesteryl ester transfer process by which cholesteryl esters are transferred from HDL towards triglyceride-rich lipoproteins and a decreased ability of high density lipoproteins (HDL) to protect oxidative modification of LDL *in vitro* are more pronounced in the context of Type 2 diabetes mellitus (T2DM) [19,20]. Additionally, low-normal thyroid function may confer decreased circulating levels of the natural anti-oxidant, bilirubin in T2DM and in insulin resistant individuals [21,22]. Such possible effect modification of chronic hyperglycemia or insulin resistance on the relationship of a number of cardio-metabolic biomarkers with low-normal thyroid function makes it relevant to assess whether the association of lipoprotein subfraction distribution with thyroid function varies according to diabetes status.

The present study was initiated to i) evaluate in subjects with and without T2DM whether low-normal thyroid function confers altered lipoprotein subfraction levels, measured by nuclear magnetic resonance (NMR) spectroscopy, and ii) to determine the extent to which such possible relationships are modified in T2DM.

Materials and Methods

Subjects

The study was performed in a University Hospital setting, and was approved by the medical ethics committee of the University Medical Center Groningen, The Netherlands. Caucasian participants (aged >18 years) were recruited by advertisement, and provided written informed consent. T2DM was previously diagnosed by primary care physicians using guidelines from the Dutch College of General Practitioners (fasting plasma glucose ≥7.0 mmol/L and/or non-fasting plasma glucose ≥11.1 mmol/L). T2DM patients had been given dietary advice. T2DM patients who were treated with metformin and/or sulfonylurea were eligible, but patients using other glucose lowering drugs and/or insulin were not allowed to participate. The use of anti-hypertensive medication was allowed. Eligible subjects had a serum TSH as well as a serum free thyroxine (free T_4) level within the institutional reference range (see below). Additional exclusion criteria were clinically manifest cardiovascular disease, renal insufficiency (elevated serum creatinine and⁄or urinary albumin >20 mg/L), liver disease (serum transaminase levels >2 times the upper reference limit), pregnancy and use of lipid lowering drugs. Subjects who used other medications (except for oral contraceptives), current smokers and subjects who used >3 alcoholic drinks daily (one drink was assumed to contain 10 grams of alcohol) were also excluded.

Physical examination did not reveal pulmonary or cardiac abnormalities. Body mass index was calculated as weight (kg) divided by height (m) squared. Waist circumference was measured on the bare skin between the 10th rib and iliac crest. Blood pressure was measured after 15 min of rest at the left arm using a sphygmomanometer. The participants were evaluated between 0800 and 1000 h after an overnight fast.

Laboratory analyses

Serum and EDTA-anticoagulated plasma samples were stored at -80 ^{0}C until analysis. Plasma glucose and glycated hemoglobin (HbA1c) levels were measured shortly after blood collection.

Serum TSH (sandwich principle; Roche Diagnostics GmbH., Mannheim, Germany, cat. no. 117314591; reference range 0.5-4.0 mU/L) and free thyroxine (free T_4; competition principle; Roche Diagnostics GmbH., Mannheim Germany, cat. no. 11731297; reference range 11.0-19.5 pmol/L) were measured by electrochemiluminescence immunoassay using a Modular Analytics immunoassay analyzer.

Plasma total cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos 11875540 and 11876023, respectively; Roche Diagnostics GmbH, Mannheim, Germany). HDL cholesterol was measured with a homogeneous enzymatic colorimetric test (Roche/Hitachi, cat no 04713214; Roche Diagnostics GmbH,

Mannheim, Germany). Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. Low density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula if the triglyceride concentration was <4.5 mmol/L. ApoA-I and apoB were assayed by immunoturbidimetry (Roche/Cobas Integra Tina-quant catalog no. 03032566 and 033032574, respectively, Roche Diagnostics).

VLDL, LDL and HDL particle profiles were measured by nuclear magnetic resonance (NMR) spectroscopy with the LipoProfile-3 algorithm (LipoScience Inc., Raleigh, North Carolina, USA), as described [28]. VLDL, LDL and HDL subclasses were quantified from the amplitudes of their spectroscopically distinct lipid methyl group NMR signals, and were expressed in concentration units, i.e. µmol/L or nmol/L. The lipoprotein subfraction particle concentrations are considered to represent an estimate of the respective lipoprotein particle numbers [21]. Diameter range estimates were for VLDL: large VLDL (including chylomicrons if present; >60 nm), medium VLDL (35 to 60 nm) and small VLDL (27 to 35 nm), for LDL: IDL (23 to 27 nm), large LDL (21.2 to 23 nm) and small LDL (18 to 21.2 nm), and for HDL: large HDL particles: 9.4 to 14 nm; medium HDL particles: 8.2 to 9.4 nm; small HDL particles: 7.3-8.2 nm. The VLDL, LDL and HDL particle concentrations were calculated as the sum of the respective lipoprotein subclasses. Weighted-average VLDL, LDL and HDL sizes were derived from the sum of the diameter of each subclass multiplied by its relative mass percentage based on the amplitude of its methyl NMR signal [23].

Serum aminotransferase (ALT) was measured with pyridoxal phosphate activation (Merck MEGA, Darmstadt, Germany). Standardization was performed according to International Federation of Clinical Chemistry guidelines. The upper reference value applied for ALT was 30 U/L. Glucose was analyzed with an APEC glucose analyzer (APEC Inc., Danvers, MA). HbA1c was measured by high-performance liquid chromatography (Bio-Rad, Veenendaal, the Netherlands; normal range: 27-43 mmol/mol). Plasma non-esterified fatty acids (NEFA) were measured using an enzymatic colorimetric method (Wako Chemicals, Neuss, Germany, cat no 43691995).

The intra-assay and inter-assay coefficients of variation of TSH, free T_4, ALT, NEFA, lipids, (apo)lipoproteins, and VLDL, LDL and HDL subfraction measurements were ≤7 % and ≤8 %, respectively.

Statistical analysis

SPSS 20 (version 20.0, SPSS Inc., Chicago, IL, USA) was used for data analysis. Data are expressed as means ± SD, medians (interquartile ranges) or in numbers. Differences between subjects with and without T2DM were determined by unpaired T-tests, Mann–Whitney U tests or Chi-square tests where appropriate.

Serum TSH and free T_4 levels were normally distributed (Kolgomorov–Smirnov test: p=0.74 and p=0.31, respectively). Since triglycerides, several lipoprotein subfraction characteristics and ALT were not parametrically distributed, these variables were logarithmically

transformed for correlation analyses. Univariate relationships were calculated using Pearson correlation coefficients.

Multivariable linear regression analyses were carried out to disclose the independent relationships of plasma (apo)lipoproteins and lipoprotein subfraction characteristics with thyroid function parameters. In addition, multivariable linear regression analyses were performed to determine interactions of diabetes status with thyroid function parameters impacting on plasma (apo)lipoproteins and lipoprotein subfraction characteristics. Interaction terms were calculated as the product terms of TSH or free T_4 with the presence of T2DM. To account for possible outliers the distributions of TSH and free T_4 were centered to their mean value by subtracting the individual value from the group mean value. Interaction terms were considered to be statistically significant at two-sided *p*-values <0.10 [24,25]. Otherwise, the level of significance was set at two-sided *p*-values <0.05.

Results

Out of 123 potentially eligible subjects, 61 T2DM patients and 52 non-diabetic control subjects were included in the study (Table 1). Ten subjects were excluded because of either a TSH or a free T_4 outside the reference range. In T2DM subjects diabetes duration was 5.0 (4.0-6.4) years. Fourteen T2DM patients used metformin and 11 patients used sulfonylurea alone, whereas both drugs were used by 18 patients. Other glucose lowering drugs were not used. Anti-hypertensive medication (mostly angiotensin-converting enzyme inhibitors, angiotensin II antagonists and diuretics, alone or in combination) were used by 25 subjects with T2DM and by none of the non-diabetic subjects (p<0.001). One pre-menopausal and 2 post-menopausal women without T2DM used estrogens. T2DM subjects were older and were more likely to be men (Table 1). Blood pressure, BMI, waist circumference, HbA1c, plasma glucose, serum ALT activity and plasma NEFA levels were also increased in T2DM subjects. TSH and free T_4 levels were not different between T2DM and non-diabetic subjects (Table 1).

Table 1. Clinical characteristics, metabolic control, alanine amino transferase (ALT), non-esterified free fatty acids (NEFA) and thyroid function parameters in 61 subjects with Type 2 diabetes mellitus (T2DM) and in 52 non-diabetic subjects.

	T2DM subjects (n=61)	**Non-diabetic subjects** (n=52)	*p*-value	*p*-value*
Age (years)	58 ± 9	54 ± 10	0.013	
Sex (men/women)	39/22	22/30	0.035	
Systolic blood pressure (mm Hg)	144 ± 20	131 ± 20	0.001	0.007
Diastolic blood pressure (mm Hg)	87 ± 9	83 ± 12	0.034	0.068
BMI (kg/m^2)	28.8 ± 4.8	25.9 ± 4.2	<0.001	<0.001
Waist circumference (cm)	102 ± 14	87 ± 13	<0.001	<0.001
Plasma glucose (mmol/L)	9.0 ± 2.4	5.7 ± 0.6	<0.001	<0.001
HbA1c (mmol/mol)	50 ± 8	34 ± 3	<0.001	<0.001
ALT (U/L)	33 (25-71)	22 (17-26)	<0.001	<0.001
NEFA (µmol/L)	343 ± 102	288 ± 98	0.005	<0.001
TSH (mU/L)	1.56 ± 0.73	1.68 ± 0.65	0.33	0.69
Free T_4 (pmol/L)	13.9 ± 1.4	13.7 ± 1.5	0.31	0.42

Data are means ± SD and medians (interquartile ranges) and numbers. BMI: body mass index; free T4: free thyroxine; HDL: high density lipoproteins; TSH: thyroid-stimulating hormone; p-value: p-value after adjustment for age and sex.*

Plasma total cholesterol was lower in T2DM subjects, but non-HDL cholesterol, LDL cholesterol and apoB levels were not significantly different between T2DM and non-diabetic subjects (Table 2). HDL cholesterol and apoA-I were lower coinciding higher triglycerides in T2DM subjects. The between group differences in these variables were essentially similar after age and sex adjustment, except for apoA-I for which the differences did not reach statistical significance (Table 2). The VLDL particle concentration was not different between T2DM and non-diabetic subjects, but large VLDL particles were increased in T2DM (Table 2). The LDL particle concentration was increased in T2DM, but the difference with non-diabetic subjects was no longer present after age and sex adjustment. Small LDL particles were increased in T2DM. The HDL particle concentration was similar in T2DM and non-diabetic subjects. Large and medium HDL particles were decreased, and small HDL particles were increased in T2DM, although the differences with non-diabetic subjects did not reach significance after age and sex adjustment. Furthermore, VLDL particle size was increased, whereas LDL and HDL particle size were decreased in T2DM.

Table 2. Plasma lipids, apolipoproteins (apos), as well as very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) subfraction characteristics in 61 subjects with Type 2 diabetes mellitus (T2DM) and in 52 non-diabetic subjects.

	T2DM subjects (n=61)	**Non-diabetic subjects** (n=52)	*p*-value	*p*-value*
Total cholesterol (mmol/L)	5.35 ± 0.90	5.70 ± 0.94	0.050	0.045
Non-HDL cholesterol (mmol/L)	4.09 ± 1.00	4.15 ± 0.99	0.76	0.49
LDL cholesterol (mmol/L)	3.24 ± 0.82	3.24 ± 0.82	0.21	0.105
LDL cholesterol (mmol/L)	3.24 ± 0.82	3.24 ± 0.82	0.21	0.105
HDL cholesterol (mmol/L)	1.27 ± 0.39	1.56 ± 0.41	<0.001	0.002
Triglycerides (mmol/L)	1.82 (1.20-2.39)	1.34 (0.88-1.89)	0.020	0.050
ApoB (g/L)	0.92 ± 0.22	0.93 ± 0.23	0.83	0.49
ApoA-I (g/L)	1.35 ± 0.23	1.45 ± 0.23	0.022	0.092
VLDL particle concentration (nmol/L)	72 (50-92)	61 (49-101)	0.43	0.97
Large VLDL (nmol/L)	7.3 (2.7-11.4)	3.3 (2.0-7.5)	0.007	0.019
Medium VLDL (nmol/L)	30.1 (15.2-41.9)	25.3 (13.2-42.8)	0.61	0.95
Small VLDL (nmol/L)	28.1 (16.7-44.2)	34.0 (21.2-44.5)	0.32	0.14
LDL particle concentration (nmol/L)	1264 (1060-1499)	1123 (942-1368)	0.043	0.16
IDL (nmol/L)	192 (124-248)	191 (145-279)	0.30	0.18
Large LDL (nmol/L)	473 (335-586)	507 (432-644)	0.14	0.069
Small LDL (nmol/L)	637 (436-896)	363 (246-665)	0.001	0.014
HDL particle concentration (µmol/L)	33 (29-37)	34 (32-36)	0.18	0.51
Large HDL (µmol/L)	3.4 (2.2-5.9)	5.5 (3.0-9.0)	0.009	0.12
Medium HDL (µmol/L)	10.5 (6.5-13.7)	12.4 (10.0-16.0)	0.012	0.066
Small HDL (µmol/L)	18.4 (14.9-21.1)	15.5 (11.6-18.0)	0.002	0.105
VLDL particle size (nM)	51.1 (45.6-58.4)	44.2 (41.8-50.5)	<0.001	0.001
LDL particle size (nM)	20.7 (20.3-21.3)	21.3 (20.9-21.5)	0.002	0.004
HDL particle size (nM)	8.8 (8.6-9.2)	9.2 (8.7-9.6)	0.004	0.035

Data are means ± SD and medians (interquartile ranges). IDL: intermediate density lipoproteins; non-HDL: non-high density lipoproteins; LDL cholesterol was calculated in 58 T2DM subjects and in 50 non-diabetic subjects. p-value: p-value after adjustment for age and sex.*

In the combined subjects, the univariate relationships of plasma cholesterol, non-HDL cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, apoB and apoA-I with either TSH or free T_4 did not reach significance. In T2DM subjects plasma triglycerides were correlated positively with TSH (Table 3). Furthermore in the combined subjects, large VLDL particles were correlated inversely, whereas small VLDL particles were correlated positively with free T_4. In T2DM subjects, large VLDL particles were correlated positively with TSH and inversely with free T_4. Except for a positive correlation of medium HDL particles with TSH in non-diabetic subjects, there were no significant relationships of LDL and HDL subfraction characteristics with thyroid function status. In the combined subjects, as well as in the T2DM and non-diabetic subjects separately, VLDL particle size was correlated inversely with free T_4. In T2DM subjects, there was also a positive relationship of VLDL particle size with TSH.

Table 3. Univariate correlations of plasma lipids, apolipoproteins, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) subfraction characteristics with thyroid function parameters in 61 subjects with Type 2 diabetes mellitus (T2DM) and in 52 non-diabetic subjects.

	All subjects combined (n=113)		**T2DM subjects** (n=61)		**Non-diabetic subjects** (n=52)	
	TSH	Free T_4	TSH	Free T_4	TSH	Free T_4
Total cholesterol	0.169	-0.024	0.220	-0.077	0.077	0.069
LDL cholesterol	0.098	0.033	0.092	0.00	0.071	0.097
Non-HDL cholesterol	0.159	-0.027	0.249	-0.107	0.031	0.067
HDL cholesterol	-0.004	0.011	-0.133	0.097	0.097	-0.003
Triglycerides	0.167	-0.106	0.314**	-0.224	-0.027	-0.006
ApoB	0.079	-0.044	0.059	0.005	-0.091	0.003
ApoA-I	-0.005	0.035	-0.186	0.099	0.187	0.009
VLDL particle concentration	0.121	0.070	0.059	0.005	0.222	0.143
Large VLDL	0.113	-0.194*	0.257*	-0.293*	-0.010	-0.156
Medium VLDL	0.036	0.046	-0.009	-0.055	0.106	0.139
Small VLDL	0.035	0.198*	-0.156	0.185	0.350**	0.059
LDL particle concentration	-0.026	-0.035	0.114	-0.028	-0.152	-0.080
IDL	-0.089	-0.060	-0.043	-0.003	-0.188	-0.105
Large LDL	-0.037	0.104	-0.108	0.156	0.046	0.076
Small LDL	-0.096	-0.030	-0.017	-0.026	-0.135	-0.093
HDL particle concentration	-0.106	-0.046	-0.246	0.052	0.121	-0.172
Large HDL	0.022	0.087	-0.089	0.229	0.111	-0.003
Medium HDL	0.045	-0.067	-0.100	-0.015	0.281*	-0.097
Small HDL	-0.161	0.002	-0.108	0.049	-0.182	-0.071
VLDL size	0.053	-0.261**	0.291*	-0.339**	-0.197	-0.282*
LDL size	-0.054	0.099	-0.196	0.116	0.074	0.157
HDL size	-0.098	0.016	0.089	0.073	0.063	0.020

*Pearson correlation coefficients are shown. Free T4: free thyroxine; TSH: thyroid-stimulating hormone. Triglycerides, as well as VLDL, LDL and HDL subfraction characteristics are logarithmically transformed. *LDL cholesterol was calculated in 58 T2DM subjects and in 50 non-diabetic subjects. *$p < 0.05$; **$p \leq 0.02$; ***$p \leq 0.01$.*

Multivariable linear regression analyses were carried out to determine the independent contributions of TSH and free T_4 to plasma triglycerides, large VLDL particles and VLDL size, representing those variables that were univariately correlated with these thyroid function parameters in either T2DM subjects, non-diabetic subjects or in all subjects combined. In all subjects combined, plasma triglycerides were related positively to TSH, taking account of age, sex and diabetes status (Table 4, models 1). The relationship of triglycerides with free T_4 was not significant (Table 4, models 1). Large VLDL particles were related inversely to free T_4 and tended to be related positively to TSH (Table 4, models 2). VLDL size was related inversely to free T_4, but not significantly to TSH (Table 4, models 3). The relationships of plasma triglycerides with TSH (β=0.172, *p*=0.073 and β=0.172, *p*=0.088), of large VLDL particles with free T_4 (β=-0.200, *p*=0.035 and β=-0.212, *p*=0.023) and of VLDL size with free T_4 (β=-0.283, *p*=0.002 and β=-0.293, *p*=0.001) remained essentially similar after additional adjustment for the use of antihypertensive medication or glucose lowering drugs (data not shown). In further analyses, the relationships of plasma triglycerides with TSH (β=0.180, *p*=0.047), of large VLDL particles with free T_4 (β=-0.181, *p*=0.048) and of VLDL size with free T_4 (β=-0.221, *p*=0.011) remained present after additional adjustment for BMI (for BMI: β=0.326, *p*<0.001, β=0.360, *p*<0.001 and β=0.289, *p*=0.002, respectively; data not shown). The relationships of plasma triglycerides with TSH (β=0.204, *p*=0.028), of large VLDL particles with free T_4 (β=-0.217, *p*=0.014) and of VLDL size with free T_4 (β=-0.286, *p*=0.001) were also independent of relationships of plasma NEFA and serum ALT (for NEFA: β=0.220, *p*=0.039, β=0.307, *p*=0.003 and β=0.250, *p*=0.009, respectively; for ALT: β=0.143, *p*=0.17, β=0.125, *p*=0.21 and β=0.201, *p*=0.35, respectively; data not shown).

In view of the univariate relationships of plasma triglycerides and several VLDL subfraction characteristics in T2DM subjects only, we next determined whether the relationships of plasma triglycerides, large VLDL particles and VLDL size with thyroid function parameters were modified by diabetes status. Multivariable linear regression analyses did not reveal significant interactions of either TSH with the presence of T2DM on plasma triglycerides (β=0.247, *p*=0.104), of free T_4 with T2DM on large VLDL particles (β=-0.077, *p*=0.57) or of free T_4 with T2DM on VLDL particle size (β=-0.059, *p*=0.63; data not shown).

Table 4. Multivariable linear regression analyses demonstrating independent relationships of thyroid function parameters with plasma triglycerides, large very low density particle (VLDL) particles and VLDL particle size in 61 subjects with Type 2 diabetes mellitus (T2DM) and in 52 non-diabetic subjects combined.

	Models 1 Triglycerides				Models 2 Large VLDL particles				Models 3 VLDL size			
	β	*p*-value	β	*p*-value	β	*p*-value	β	*P*-value	β	*p*-value	β	*p*-value
Age	-0.009	0.92	-0.017	0.87	-0.005	0.96	0.005	0.96	-0.069	0.46	-0.043	0.63
Sex (men/women)	0.117	0.226	0.088	0.37	0.165	0.084	0.132	0.16	0.162	0.083	0.132	0.14
T2DM (yes/no)	0.195	0.046	0.200	0.044	0.235	0.015	0.246	0.010	0.319	0.001	0.337	<0.001
TSH	0.196	0.039			0.159	0.09			0.096	0.29		
Free T_4			-0.121	0.21			-0.215	0.020			-0.285	0.001

β: standardized regression coefficient; free T4: free thyroxine; TSH: thyroid-stimulating hormone.
Plasma triglycerides, large VLDL particles and VLDL particle size are logarithmically transformed.
Models 1: *triglycerides as dependent variable*
Models 2: *large VLDL particles as dependent variable*
Models 3: *VLDL size as dependent variable*

Discussion

This study has shown univariate relationships of plasma triglycerides, large VLDL particles and VLDL particle size with low-normal thyroid function in euthyroid T2DM subjects. In non-diabetic subjects, VLDL particle size was inversely correlated with free T_4. Of note, the relationships of triglycerides, large VLDL particles and VLDL particle size with low-normal thyroid function were not to a significant extent modified in the context of T2DM. In the combined subjects, multivariable linear regression analysis demonstrated that plasma triglycerides were related positively to TSH, whereas large VLDL particles and VLDL particle size were related inversely to free T_4 taking account of age, sex and diabetes status. The present results are, therefore, consistent with the concept that variations in thyroid function even in the low-normal range may contribute to higher circulating triglycerides consequent to increased large VLDL particles.

The modest positive relationship of plasma triglycerides with TSH that was documented by multivariable linear regression analysis in the combined subjects extends comparable yet not unequivocally documented observations in large scale studies among euthyroid subjects recruited from the general population [6-10]. Besides higher plasma triglycerides and lower levels of HDL cholesterol, expected abnormalities in lipoprotein subfraction distribution, including predominance of large VLDL and small LDL particles were observed in T2DM subjects [17,26]. In the current study, TSH and free T_4 levels were not different between T2DM and non-diabetic subjects. In other reports, free T_4 levels were unchanged [20,27] or slightly higher in T2DM [19]. Of further interest, metformin administration could affect pituitary-thyroid hormone feedback regulation. However, a meta-analysis has shown that metformin may lower the TSH level in hypothyroid but not in euthyroid subjects [28], whereas no independent effect of metformin therapy on the TSH level was found in a survey among T2DM subjects [29]. In our study, the relationships of plasma triglycerides and VLDL characteristics with low-normal thyroid function remained significant after additional adjustment for metformin treatment.

Increased hepatic production of large VLDL particles is considered to represent an important mechanism responsible for higher plasma triglycerides, as observed in T2DM, obesity and the metabolic syndrome [30-32]. Clearly, the presently demonstrated relationships of large VLDL and VLDL particle size with low-normal thyroid function are in line with recent data showing that the hepatic production of large VDL particles is elevated in subclinical hypothyroidism [14]. Given the strong contribution of large VLDL particles to the total plasma triglyceride concentration [33], it is also likely that an increased concentration/particle numbers of large VLDL is relevant for higher plasma triglycerides associated with low-normal thyroid function. Furthermore, multivariable linear regression analysis demonstrated that plasma triglycerides, large VLDL particles an VLDL size were positively related to BMI and to the plasma NEFA concentration, as a proxy of its rate

of appearance [30,34]. These results would agree with the concept that adiposity, which contributes to enhanced NEFA delivery to the liver, is a determinant of hepatic VLDL production [30-32]. On the other hand, the relationship of low-normal thyroid function with VLDL particle characteristics was not to a considerable extent explained by BMI or NEFA levels. In comparison, plasma NEFA levels and its rate of appearance were found to be unchanged in subclinical hypothyroidism [14]. In the present study, we did not observe a relationship of serum ALT activity with VLDL subfraction characteristics. Although hepatic fat accumulation is regarded as driving force for enhanced VLDL secretion [32,35], it remains uncertain whether serum ALT activity, which we used as a surrogate of hepatic fat accumulation [36-38], was sensitive enough to discern relationships with VLDL particle characteristics.

Previous findings have underscored that the relationship of low-normal thyroid function with biologically plausible atherogenic changes in (lipoprotein-related) cardiometabolic biomarkers may be modified in the context of chronic hyperglycemia or insulin resistance [19-22]. These findings provided a rationale to compare thyroid function-lipoprotein subfraction relationships between T2DM and non-diabetic subjects. We could not demonstrate significant effect modification of the presence of T2DM on the relationship of plasma triglycerides and VLDL subfraction characteristics with low-normal thyroid function. The degree of hyperglycemia was moderate in most of the participating T2DM subjects. In addition, we excluded subjects who were using lipid lowering drugs before entry in the study. Thus, T2DM subjects with modest lipoprotein abnormalities were preferentially included. It is possible that these subject characteristics could have masked diabetes-associated modifications in the relationship of lipoprotein subfraction characteristics with low-normal thyroid function.

The precursor-product relationship between VLDL and LDL is well established [30,39]. Through concerted actions of cholesteryl ester transfer protein and lipases large VLDL particles play a pivotal role in the generation of small dense LDL, which are prone to oxidative modification [40]. Thyroid hormones are involved in the regulation of cholesteryl ester transfer protein and lipases [41,42]. Furthermore, increased LDL oxidation is inversely associated with thyroid function [43]. Increased levels of large VLDL particles probably play a pathogenetic role in enhanced atherosclerosis susceptibility [30,39]. Nonethleless, it is obvious that the extent to which increased large VLDL particles could particularly influence atherosclerosis susceptibility in the context of low-normal thyroid function is uncertain at present.

Several other methodological aspects and limitations of our study need to be considered. First, the rather small study population underscores the need to replicate our findings in a large cohort. Second, we carried out a cross-sectional study, making that cause-effect relationships cannot be established with certainty. Third, for logistic reasons we only measured free T_4. Measurement of free T_3 could have yielded additional information.

However, thyroid hormone effects on apoB-containing lipoproteins during reversal of both hypo- and hyperthyroidism to euthyroidism appear to be sufficiently documented by free T_4 measurement alone [44]. Moreover, differences in free T_3 in association with variations in TSH levels within the euthyroid range are less pronounced than changes in free T_4 [9]. Fourth, we employed a highly reproducible NMR spectroscopy analysis to determine lipoprotein subfraction characteristics [23,33], but some discrepancies with more conventional lipoprotein subfraction assays cannot be excluded [45].

In conclusion, this study suggests that low-normal thyroid function may confer increased plasma triglycerides, large VLDL particles and increased VLDL particle size.

Conflict of interest

This study is investigator driven. The authors state no conflict of interest.

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4.

Higher plasma apoE levels are associated with low-normal thyroid function: studies in diabetic and non-diabetic subjects

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Abstract

Low-normal thyroid function within the euthyroid range may confer higher plasma triglycerides, but relationships with plasma apolipoprotein (apo) E, which plays an important role in the metabolism of triglyceride-rich apoB-containing lipoproteins, are unknown. We determined relationships of plasma apoE with thyroid stimulating hormone (TSH) and free thyroxine (free T_4) in euthyroid subjects with and without Type 2 diabetes mellitus (T2DM). TSH, free T_4, lipids and apoE were measured in fasting plasma from 72 T2DM subjects and 82 non-diabetic subjects. The *APOE* genotype was also determined. Free T_4 was slightly higher in T2DM (p=0.030), but TSH levels were not different vs. non-diabetic subjects. The APOE genotype distribution was not different between the groups. None of the participants had the ε2/ε2 genotype. Plasma triglycerides were higher in T2DM (p=0.037). ApoB and apoE levels were not different between the groups. In all subjects combined, multivariable analysis showed that plasma triglycerides (p=0.039), non-high density lipoprotein (non-HDL) cholesterol (p=0.030) and apoE levels (p=0.002) were each independently and positively associated with TSH after adjustment for age, sex, T2DM and the presence of the *APOE* ε3 allele. Furthermore, the associations of TSH with apoE remained present after adjustment for either triglycerides, non-HDL cholesterol or apoB (p=0.005 to p=0.023). The presence of T2DM did not modify the relationships of TSH with these (apo)lipoprotein variables (p=0.11 to p=0.36). In conclusion, low-normal thyroid function, as indicated by higher TSH levels within the euthyroid range, may influence the metabolism of triglyceride-rich lipoproteins by affecting apoE regulation.

Introduction

Apolipoprotein E (apoE) is a multifunctional apolipoprotein which is produced by several tissues including the liver [1-3]. Its function in receptor-mediated uptake of very low density lipoproteins (VLDL), the main circulating triglyceride-rich lipoprotein in the fasting state, is well appreciated [1,3]. ApoE also plays an important role in hepatic VLDL overproduction and in impaired VLDL clearance [4,5]. In line, the plasma apoE concentration is strongly correlated with triglycerides, and is elevated in subjects with the metabolic syndrome (MetS) [2,6,7,8]. In addition, apoE exerts anti-oxidative and anti-inflammatory effects [2,9]. Although apoE has a predominant anti-atherogenic role in animal models [1-3,10], plasma apoE levels have been documented to be associated positively with incident cardiovascular disease (CVD) in elderly subjects, and in women with elevated high density lipoprotein (HDL) cholesterol in combination with high C-reactivity protein (CRP) levels [11-14]. It seems, therefore, plausible that higher total plasma apoE levels may reflect increased CVD risk in humans.

Considerable attention has been paid to the effect of subclinical hypothyroidism (SCH), i.e. an elevated thyroid-stimulating hormone (TSH) level in the context of a free thyroxine (free T_4) concentration within the euthyroid range, on atherosclerosis development. SCH could to some extent predict higher risk of atherosclerotic CVD, and may relate to increased carotid artery intima media (cIMT) thickening [15,16]. In addition, an association of lower free T_4 levels within the euthyroid range with increased cIMT has been observed [17,18]. Moreover, both SCH, and a low-normal thyroid functional status, as indicated by a high-normal TSH or a low-normal free T_4 level within the euthyroid range, confer unfavourable plasma lipoprotein and lipid changes such as higher triglyceride levels [16,19]. In this context it is relevant that SCH is featured by increased hepatic production of large VLDL particles [20]. Remarkably, apoE gene expression in rat liver as well as its secretion by HepG2 cells may be inhibited by thyroid hormone [21,22]. Taken together, it is plausible to hypothesize that a low-normal thyroid function status relates to higher plasma apoE levels which in turn may affect the metabolism of triglyceride-rich lipoproteins.

In the present report we aimed to examine whether low-normal thyroid function relates to alterations in plasma apoE levels. Given the prominent role of increased hepatic VLDL production in diabetic dyslipidemia [23-25] our study was carried out in subjects with and without Type 2 diabetes mellitus (T2DM).

Materials and Methods

Study design and subjects

The study was performed in a University Hospital setting, and was approved by the medical ethics committee of the University Medical Center Groningen, The Netherlands. Caucasian participants (aged >18 years) were recruited by advertisement, and provided written informed consent. T2DM was previously diagnosed by primary care physicians using guidelines from the Dutch College of General Practitioners (fasting plasma glucose ≥7.0 mmol/l and/or non-fasting plasma glucose ≥11.1 mmol/l). T2DM patients had been given dietary advice via their primary care physicians according to Dutch College of General Practitioners guidelines but precise data on diet composition of the individual participants were not available. T2DM patients who were treated with metformin and/or sulfonylurea were eligible. Patients using other glucose lowering drugs and/or insulin were not allowed to participate in order to minimize bias due to advanced diabetes-induced metabolic derangement, and to obviate effects of exogenous insulin on hepatic lipoprotein metabolism [23]. The use of anti-hypertensive medication was allowed. Eligible subjects had a serum TSH as well as a serum free thyroxine (free T_4) level within the institutional reference range (see below). Additional exclusion criteria were clinically manifest cardiovascular disease, renal insufficiency (estimated glomerular filtration rate < 60 ml/min/1.73 m^2 and/or urinary albumin >20 mg/l), liver disease (serum transaminase levels >2 times the upper reference limit), pregnancy and use of lipid lowering drugs. The use of other medications (except for oral contraceptives) was an exclusion criterion. Current smokers and subjects who used >3 alcoholic drinks daily (one drink was assumed to contain 10 grams of alcohol) were also excluded to obviate confounding due to smoking and excessive alcohol consumption on lipoprotein metabolism.

Physical examination did not reveal pulmonary or cardiac abnormalities. Body mass index was calculated as weight (kg) divided by height (m) squared. Waist circumference was measured on the bare skin between the 10th rib and iliac crest. Blood pressure was measured after 15 min of rest at the left arm using a sphygmomanometer. The participants were evaluated between 0800 and 1000 h after a 10 hour fast.

Laboratory measurements

Serum and EDTA-anticoagulated plasma samples were stored at -80 ^{0}C until analysis. Plasma glucose and glycated hemoglobin (HbA1c) levels were measured shortly after blood collection.

Serum TSH (sandwich principle; Roche Diagnostics GmbH., Mannheim, Germany, cat. no. 117314591; reference range 0.5-4.0 mU/l) and free T_4 (competition principle; Roche Diagnostics GmbH, Mannheim Germany, cat. no. 11731297; reference range 11.0-19.5 pmol/l) were measured by electrochemiluminescence immunoassay using a Modular Analytics immunoassay analyzer.

Plasma total cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos 11875540 and 11876023, respectively; Roche Diagnostics GmbH, Mannheim, Germany). HDL cholesterol was measured with a homogeneous enzymatic colorimetric test (Roche/Hitachi, cat no 04713214; Roche Diagnostics GmbH, Mannheim, Germany). Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. Low density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula if the triglyceride concentration was <4.5 mmol/l. Apolipoprotein B100 (apoB) was assayed by immunoturbidimetry (Roche/Cobas Integra Tina-quant catalog no. 033032574; Roche Diagnostics). ApoE was measured using an immunoturbidimetric assay (Wako Inc, Osaka, Japan; catalog no. 417-35906). The intra-assay and inter-assay coefficients of variation of TSH, free T_4, lipids and apos were <5 % and <6 %, respectively.

APOE genotypes (rs429358 and rs7412) were determined by allelic discrimination on a CFX system (Bio Rad), using predesigned primers C-3084793-20 and C-904973-10 and Taqman Universal PCR mastermix (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). To this end DNA was extracted from whole blood using the Qiampmini kit (Qiagen). The method has been validated against a previously described restriction isotyping procedure [26,27].

Statistical analysis

SPSS 22 (version 22.0, SPSS Inc., Chicago, IL, USA) was used for data analysis. Data are expressed as means ± SD, medians (interquartile ranges) or in numbers. Differences between subjects with and without T2DM were determined
by unpaired T-tests or Chi-square tests. *APOE* genotype distribution between diabetic and non-diabetic subjects was compared by multinomial Chi-square test. Since triglycerides, TSH and free T_4 levels were not parametrically distributed, these variables were logarithmically transformed to compare between group differences, as well as for correlation analyses. Univariate relationships were calculated using Pearson correlation coefficients.

Multivariable linear regression analyses were carried out to disclose the independent relationships of plasma (apo)lipoproteins with TSH levels. We also determined whether the relation of TSH with plasma apoE and other (apo)lipoprotein variables was different in diabetic and non-diabetic subjects. To this end interaction terms were calculated as the product terms of TSH with the presence of T2DM. Interaction terms were considered to be statistically significant at two-sided p-values <0.10 [28]. Otherwise, the level of significance was set at two-sided p-values <0.05.

Results

Fourteen of a total of potentially eligible 168 subjects were excluded because of either a TSH or a free T_4 level outside the reference range. The study population was comprised of 72 T2DM patients and 82 non-diabetic control subjects (**Table 1**). Among T2DM subjects diabetes duration was 5.4 (4.0-6.5) years. Fifteen T2DM subjects used metformin and 15 subjects used sulfonylurea alone, whereas another 24 subjects used both drugs. Other glucose lowering drugs were not used. Anti-hypertensive medication (mostly angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists and diuretics, alone or in combination) were used by 30 subjects with T2DM and by none of the non-diabetic subjects ($p<0.001$). One pre-menopausal and 2 post-menopausal women were using estrogens.

T2DM subjects were older, whereas sex distribution was not different between diabetic and non-diabetic subjects (**Table 1**). Blood pressure, BMI, waist circumference, HbA1c, plasma glucose were also increased in T2DM subjects. Free T_4 levels were slightly higher in T2DM subjects but TSH levels were not different between the groups. The *APOE* genotype distribution was not different between diabetic and non-diabetic subjects (**Table 1**). None of the participants was homozygous for the ε2 allele. Plasma total cholesterol was lower in T2DM subjects, but non-HDL cholesterol, LDL cholesterol, apoB and apoE levels were not significantly different between T2DM and non-diabetic subjects (**Table 1**). HDL cholesterol was lower coinciding with higher triglycerides in T2DM subjects.

Table 1. Clinical characteristics, thyroid function parameters, plasma lipids, lipoproteins, apolipoprotein B (apoB), apolipoprotein E (apoE) in subjects with 72 Type 2 diabetes mellitus (T2DM) and in 82 non-diabetic subjects.

	T2DM subjects (n=72)	**Non-diabetic subjects** (n=82)	p-value
Age (years)	59 ± 9	55 ± 10	0.029
Sex (men/women)	47/25	47/35	0.31
Systolic blood pressure (mm Hg)	143 ± 20	131 ± 19	<0.001
Diastolic blood pressure (mm Hg)	87 ± 9	82 ± 11	0.009
BMI (kg/m^2)	28.4 ± 4.6	26.0 ± 3.8	0.001
Waist circumference (cm)	100 ± 14	89 ± 13	<0.001
Plasma glucose (mmol/l)	9.0 ± 2.3	5.7 ± 0.7	<0.001
HbA1c (mmol/mol)	51 ± 8	40 ± 3	<0.001
TSH (mU/l)	1.38 (0.96-1.94)	1.55 (1.27-2.06)	0.11
Free T_4 (pmol/l)	14.03 (12.91-15.08)	13.38 (12.54-14.69)	0.030
APOE genotype ε2/ε3 ε3/ε3 ε3/ε4 ε2/ε4 ε4/ε4	 6 42 20 1 3	 3 62 15 0 2	0.19
Total cholesterol (mmol/l)	5.41 ± 0.91	5.72 ± 0.96	0.037
Non-HDL cholesterol (mmol/l)	4.17 ± 0.97	4.24 ± 1.02	0.68
LDL cholesterol (mmol/l)	3.30 ± 0.78	3.53 ± 0.86	0.094
HDL cholesterol (mmol/l)	1.24 ± 0.35	1.49 ± 0.41	<0.001
Triglycerides (mmol/l)	1.78 (1.17-2.47)	1.27 (0.89-1.92)	0.037
ApoB (g/l)	0.93 ± 0.20	0.96 ± 0.24	0.55
ApoE (g/l)	0.041 ± 0.123	0.039 ± 0.097	0.85

Data are means ± SD and medians (interquartile ranges) and numbers. Low density lipoprotein (LDL) cholesterol was calculated in 68 T2DM subjects and in 80 non-diabetic subjects. BMI: body mass index; free T4: free thyroxine; HbA1c: glycated hemoglobin; HDL: high density lipoproteins; TSH: thyroid-stimulating hormone.

In univariate analysis, plasma apoE was correlated positively with total cholesterol, non-HDL cholesterol, LDL cholesterol and even more strongly with triglycerides, and inversely with HDL cholesterol (**Table 2**). Comparable relationships were found in non-diabetic subjects, as well as in all subjects combined (**Table 2**).

Table 2. Univariate correlations of plasma apolipoprotein (apo) E with plasma lipids, lipoproteins, apoB and apoA-I in 72 subjects with Type 2 diabetes mellitus (T2DM) and 82 in non-diabetic subjects.

	T2DM subjects (n=72)	**Non-diabetic subjects** (n=82)	**All subjects combined** (n=154)
	ApoE	ApoE	ApoE
Total cholesterol	0.479***	0.401***	0.418***
Non-HDL cholesterol	0.564***	0.444***	0.497***
LDL cholesterol	0.244*	0.195	0.212**
HDL cholesterol	-0.324**	-0.168	-0.250**
Triglycerides	0.698***	0.563***	0.640***
ApoB	0.454***	0.381***	0.403***

*Pearson correlation coefficients are shown. Triglycerides are logarithmically transformed. LDL: low density lipoproteins; HDL: high density lipoproteins; non-HDL cholesterol: non-high density lipoproteins. LDL cholesterol was calculated in 68 T2DM subjects and in 80 non-diabetic subjects. *p<0.05; **p≤0.01; ***p<0.001.*

In T2DM subjects, total cholesterol, non-HDL cholesterol, triglycerides, apoB and apoE levels were correlated positively with TSH; triglycerides were inversely related to free T_4 (**Table 3**). In non-diabetic subjects, the correlations of TSH with these lipoprotein variables did not reach statistical significance (**Table 3**). In all subjects combined, total cholesterol, non-HDL cholesterol and apoE levels were correlated positively with TSH (**Table 3**).

Multivariable linear regression analyses were subsequently carried out in the combined subjects to determine the independent relationships of plasma triglycerides, non-HDL cholesterol apoB and apoE levels with TSH, taken account of age, sex, diabetes status and the *APOE* genotype (dichotomized as *ApoE* ε3 carriers vs. non-ε3 carriers). In all subjects combined, plasma triglycerides, non-HDL cholesterol and apoE levels were each independently associated with TSH, whereas there was no independent relationship of apoB with TSH (**Table 4**). These analysis also showed that non-HDL cholesterol and apoB levels were lower in *ApoE* ε3 carriers compared to non-ε3 carriers. The relationship of apoE with TSH remained significant after additional adjustment for the use of metformin, sulfonylurea and anti-hypertensive medication (data not shown; β=0.209, p=0.014; cf.

model 4, **Table 4**). In addition, the relationship of apoE with TSH was still significant taking account of either plasma triglycerides, non-HDL cholesterol or apoB (data not shown; β=0.147, p=0.023; β=0.168, p=0.021 and β=0.214, p=0.005, respectively; cf. model 4, **Table 4**).

Of note, there were no significant interactions between the presence of T2DM and TSH on plasma triglycerides (β=0.209, p=0.11; cf. model 1, **Table 4**), non-HDL cholesterol (β=0.119, p=0.36; cf. model 2, **Table 4**), apoB (β=0.172, p=0.18; cf. model 3, **Table 4**) and apoE levels (β=0.137, p=0.29; cf. model 2 **Table 4**). These results thus suggested that the relationships between TSH and these (apo)lipoprotein variables were not significantly modified in the context of T2DM.

In subsidiary analyses in which we only included *APOE* ε3/ε3 carriers only (44 T2DM subjects and in 62 non-diabetic subjects), plasma triglycerides, non-HDL cholesterol and apoE were each positively related to the TSH level in age-, sex- and diabetes status-adjusted multivariable models (**Table 5**).

Table 3. Univariate correlations of plasma lipids, lipoproteins, apolipoprotein B (apoB) and apolipoprotein E (apoE) with thyroid function parameters in 72 subjects with Type 2 diabetes mellitus (T2DM) and 82 in non-diabetic subjects.

	T2DM subjects (n=72)		**Non-diabetic subjects** (n=82)		**All subjects combined** (n=152)	
	TSH	FreeT_4	TSH	FreeT_4	TSH	FreeT_4
Total cholesterol	0.300**	-0.084	0.090	0.030	0.214**	-0.051
Non-HDL cholesterol	0.320**	-0.114	0.018	0.056	0.177*	-0.029
LDL cholesterol	0.178	0.006	0.049	0.012	0.132	-0.006
HDL cholesterol	-0.112	0.101	0.165	-0.067	0.063	-0.049
Triglycerides	0.316**	-0.247*	-0.036	0.085	0.138	-0.049
Apo B	0.282*	-0.113	-0.091	-0.014	0.094	-0.061
Apo E	0.349**	0.008	0.115	-0.132	0.240**	-0.043

*Pearson correlation coefficients are shown. LDL: low density lipoproteins; HDL: high density lipoproteins; non-HDL cholesterol: non-high density lipoproteins; Free T4: free thyroxine; TSH: thyroid-stimulating hormone. TSH, free T4 and triglycerides are logarithmically transformed. LDL cholesterol was calculated in 68 T2DM subjects and in 80 non-diabetic subjects. *p<0.05; **p≤0.01.*

Table 4. Multivariable linear regression analyses demonstrating independent relationships of plasma triglycerides, non-high density lipoprotein (non-HDL) cholesterol, apolipoprotein B (apoB) and apolipoprotein E (apoE) with thyroid-stimulating hormone (TSH) levels in 72 subjects with Type 2 diabetes mellitus (T2DM) and 82 in non-diabetic subjects combined.

	Model 1 Triglycerides		**Model 2 Non-HDL cholesterol**		**Model 3 ApoB**		**Model 4 ApoE**	
	β	p-value	β	p-value	β	p-value	β	p-value
Age	-0.107	0.19	-0.060	0.46	-0.071	0.39	-0.080	0.33
Sex (men/women)	0.122	0.13	0.132	0.10	0.172	0.033	0.055	0.49
T2DM (yes/no)	0.222	0.006	-0.026	0.75	-0.053	0.52	0.108	0.18
ApoE genotype ε3 carriers vs. non ε3 carriers	-0.088	0.27	-0.186	0.022	-0.194	0.017	-0.093	0.25
TSH	0.166	0.039	0.176	0.030	0.095	0.24	0.250	0.002

β: standardized regression coefficient. Plasma triglycerides and TSH levels are logarithmically transformed. ε3 carriers: subjects who carry at least one ApoE ε3 allele; non ε3 carriers: subjects who do not carry an ApoE ε3 allele. All models are adjusted for age, sex, diabetes status and the presence of at least one APOE ε3 allele.
Model 1: *triglycerides as dependent variable*
Model 2: *non-HDL cholesterol as dependent variable*
Model 3: *apoB as dependent variable*
Model 4: *apoE as dependent variable.*

Table 5. Multivariable linear regression analyses demonstrating independent relationships of plasma triglycerides, non-high density lipoprotein (non-HDL) cholesterol, apolipoprotein B (apoB) and apolipoprotein E (apoE) with thyroid-stimulating hormone (TSH) levels in APOE ε3/ε3 carriers only (44 subjects with Type 2 diabetes mellitus (T2DM) and in 62 non-diabetic subjects).

	Model 1 Triglycerides		**Model 2 Non-HDL cholesterol**		**Model 3 ApoB**		**Model 4 ApoE**	
	β	p-value	β	p-value	β	p-value	β	p-value
Age	-0.156	0.093	-0.100	0.32	-0.088	0.38	-0.185	0.058
Sex (men/women)	0.119	0.19	0.183	0.063	0.218	0.028	-0.051	0.69
T2DM (yes/no)	0.423	<0.001	-0.068	0.50	0.013	0.90	0.267	0.007
TSH	0.212	0.023	0.206	0.040	0.098	0.33	0.210	0.032

β: standardized regression coefficient. Plasma triglycerides and TSH levels are logarithmically transformed. All models are adjusted for age, sex and diabetes status.
Model 1: *triglycerides as dependent variable*
Model 2: *non-HDL cholesterol as dependent variable*
Model 3: *apoB as dependent variable*
Model 4: *apoE as dependent variable*

Discussion

In this report we have shown that higher plasma apoE relates to low-normal thyroid function, as evidenced by a high-normal TSH level, in euthyroid T2DM subjects. Although this relationship did not reach statistical significance in non-diabetic subjects, the association of apoE with TSH was not different in subjects with T2DM compared to non-diabetic individuals. In all subjects combined, apoE was still positively related to the TSH level in multivariable linear regression analysis in which age, sex, diabetes status and the *APOE* genotype were taken into account. Remarkably, this relationship of apoE with TSH was also present when taking account of either plasma triglycerides, non-HDL cholesterol or apoB. Our current results are, therefore, in agreement with the hypothesis that variations in thyroid function within the euthyroid range may be involved in the regulation of plasma apoE and, hence, in the metabolism of apoE-containing triglyceride-rich lipoproteins.

The positive relationships of plasma triglycerides and of non-HDL cholesterol with TSH, as demonstrated here, agree with several previous observations in population-based cohort studies (reviewed in [16]). As expected plasma apoE levels were strongly correlated with plasma triglycerides [2,6,7,8]. In hypertriglyceridemic T2DM subjects, plasma triglyceride lowering in response to atorvastatin administration coincides with a decrease in plasma apoE [29]. Despite higher plasma triglycerides, apoE was not elevated in the presently studied T2DM subjects. In comparison, plasma apoE levels were found to be elevated in more severely hypertriglyceridemic and hyperglycemic T2DM subjects in an early report [30]. Thus, it is likely that more profound dyslipidemia and/or metabolic dysregulation is required to result in plasma apoE elevations. Of note, the relationship of plasma apoE with TSH remained present when taking account of apoB-containing lipoproteins, and also of the *APOE* genotype (with apoE ε3 allele carriers expectedly having lower non-HDL cholesterol and apoB levels [31]).

Increased hepatic production of large VLDL particles in T2DM is considered to represent a pro-atherogenic abnormality which results in higher circulating triglyceride-rich lipoprotein levels [23,24]. This salient feature of diabetic dyslipidemia provided our main rationale to study T2DM subjects in the current report. Of note, subclinical hypothyroidism confers increased production of large VLDL [7], whereas predominance of large VLDL particles is associated with low-normal thyroid function [25]. Moreover, it is conceivable that thyroid function status is directly implicated in affecting apoE regulation, as previously evidenced in experimental settings [21,22]. Collectively, these data make it is plausible to postulate that the relationship of apoE with low-normal thyroid function, as documented here, may reflect a pathogenic mechanism that is involved the metabolism of (large) VLDL particles, thereby contributing to higher circulating triglyceride levels.

A number of other methodological aspects and limitations of our study need to be considered. First, the design of our study was cross-sectional, so that cause-effect relationships cannot be ascertained with certainty. Second, we only included subjects who did not use lipid lowering drugs, making it likely that T2DM subjects with relatively modest lipoprotein abnormalities were preferentially recruited. Moreover, metabolic control was adequate in most of the T2DM subjects. For these reasons, it cannot be excluded that inclusion of T2DM subjects with more outspoken lipoprotein abnormalities and/or more severe hyperglycemia could resulted in differences in the relationship of apoE with low-normal thyroid function between T2DM and non-diabetic subjects. Third, we found similar TSH levels in T2DM and non-diabetic subjects, but somewhat higher free T_4 levels in T2DM subjects as reported earlier [32]. Metformin has been proposed to affect pituitary-thyroid hormone feedback regulation, although no independent effect of metformin therapy on the TSH level was documented in euthyroid T2DM subjects [33]. In the current study, the relationship of apoE with TSH was essentially unaltered after adjustment for the use of metformin. Additionally, the relationship of apoE with TSH was also present in *APOE* ε3/ε3 carriers only, the genotype that relates to higher apoE levels [31]. Finally, it should be noted we did not assess possible effects of dietary nutrient and fat intake on plasma lipids in the context of low-normal thyroid function.

In conclusion, this study demonstrates that low-normal thyroid function, as indicated by higher TSH levels within the euthyroid range, may confer higher plasma apoE levels. It is conceivable that variation in thyroid function would influence the metabolism of triglyceride-rich apoB-containing lipoproteins by affecting apoE regulation.

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Conflict of interest
This study is investigator driven. The authors state no conflict of interest.

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5.

Pre β-HDL formation relates to high-normal free thyroxine in type 2 diabetes mellitus

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Abstract

Objectives: Low-normal thyroid function within the euthyroid range may influence plasma lipoprotein levels. Associations between variation in thyroid function and pre β-high density lipoproteins (pre β-HDL), i.e. lipid-poor or lipid free HDL particles that act as initial acceptor of cell-derived cholesterol, are unknown. We determined relationships of plasma pre β-HDL with thyroid function in euthyroid subjects with and without vs. non-diabetic subjects. HDL cholesterol and apoA-I were lower, whereas pre β-HDL (expressed as % of apoA-I), triglycerides and PLTP activity were higher in T2DM (P<0.05 to P<0.001). In T2DM, pre β-HDL formation (in apoA-I concentration and in % of apoA-I) was positively related to free T4, PLTP activity, total cholesterol and triglycerides (P<0.05 for each). Multivariable linear regression analyses, adjusted for age, sex, PLTP activity, total cholesterol and triglycerides, demonstrated that pre β-HDL formation was positively related to free T4 (in apoA-I concentration: β=0.278, P=0.014; in % of apoA-I: β=0.343, P=0.003) in T2DM, but not in non-diabetic subjects (both P>0.30; interaction terms: both P<0.05).
Conclusions: Variations in thyroid function within the euthyroid range may influence the metabolism of pre β-HDL in T2DM.

Introduction

Low-normal thyroid function, as inferred from higher TSH or lower thyroid hormone levels within the euthyroid range, may confer changes in plasma lipids and other biomarkers that relate to increased cardiovascular risk [1,2]. Single determinations of TSH and free T4 can provide relevant information regarding the effect of thyroid function status on plasma lipids and lipoproteins [2,3]. In agreement with this concept, low-normal thyroid function associates with a greater carotid intima media thickness (cIMT), an established marker of subclinical atherosclerosis [4,5]. Higher TSH levels within the euthyroid range may also predict cardiovascular mortality in women [6].

It is likely that low-normal thyroid function predicts higher plasma levels of plasma total cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides, but associations with high density lipoprotein (HDL) cholesterol have been inconsistently reported [2]. HDL particles are very heterogeneous in size, structure and composition with important consequences for their functional properties [7,8]. This underscores the relevance to discern the relationship of HDL subfractions with variations in thyroid function in more detail. In this regard, it is important that a small proportion of HDL consists of lipid poor or lipid free particles, designated pre β-HDL [9,10]. By promoting cellular cholesterol efflux, pre β-HDL particles play an important role in the reverse cholesterol transport pathway, whereby cholesterol is transported from peripheral cells back to the liver for biliary transport and excretion in the feces [8,9-11]. Although not unequivocally reported [12], higher plasma pre β-HDL concentrations are observed in subjects with cardiovascular disease [13,14]. Of further relevance, higher plasma pre β-HDL levels associate with a greater cIMT, both in diabetic and non-diabetic subjects [15,16]. It is plausible to interpret such higher pre β-HDL levels in the context of increased cardiovascular risk to be indicative of impaired conversion of pre β-HDL to more mature cholesterol-rich HDL particles, and hence to reflect impaired HDL-mediated reverse cholesterol transport [17].

Relationships of several HDL-mediated functional properties such as the cholesteryl ester transfer protein (CETP)-mediated transport of cholesteryl esters out of HDL, as well as an impaired ability of HDL to protect oxidative modification of LDL *in vitro* were recently reported to be particularly evident in individuals with Type 2 diabetes mellitus (T2DM) [18,19]. In view of the greater cIMT in conjunction with higher pre β-HDL levels in T2DM [15], it is relevant to assess whether the hitherto unexplored association of pre β-HDL lipoprotein with thyroid function varies according to diabetes status.

The present study was initiated to discern in subjects with and without T2DM whether plasma pre β-HDL is associated with variations in thyroid function within the euthyroid range. Second, we determined the extent to which such relationships are modified in the context of T2DM.

Subjects and Methods

Subjects

The study was performed in a University Hospital setting, and was approved by the medical ethics committee of the University Medical Center Groningen, The Netherlands. Caucasian participants (aged >18 years) were recruited by advertisement, and had provided written informed consent. T2DM had been previously diagnosed by primary care physicians using guidelines from the Dutch College of General Practitioners (fasting plasma glucose ≥7.0 mmol/l and/or non-fasting plasma glucose ≥11.1 mmol/l). T2DM patients who were treated with metformin and/or sulfonylurea were allowed to participate, but patients using other glucose lowering drugs and/or insulin were excluded. The use of anti-hypertensive medication was allowed. Eligible subjects had a serum TSH and a free T4 level within the institutional reference range (see below). Additional exclusion criteria were clinically manifest cardiovascular disease, renal insufficiency (estimated glomerular filtration rate < 60 ml/min/1.73 m^2 and⁄or urinary albumin >20 mg/l), liver disease (serum transaminase levels >2 times the upper reference limit), pregnancy and use of lipid lowering drugs. Subjects who used other medications (except for oral contraceptives), current smokers and subjects who used >3 alcoholic drinks daily were also excluded. Physical examination did not reveal pulmonary or cardiac abnormalities. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Blood pressure was measured after 15 min of rest at the left arm using a sphygmomanometer. The participants were evaluated between 08.00 and 10.00 h after an overnight fast.

Laboratory analyses

Serum and EDTA-anticoagulated plasma samples were stored at -80 ^{0}C until analysis. Plasma glucose and glycated hemoglobin (HbA1c) levels were measured shortly after blood collection.

Serum TSH (sandwich principle; Roche Diagnostics GmbH., Mannheim, Germany, cat. no. 117314591; reference range 0.5-4.0 mU/l) and free T4 (competition principle; Roche Diagnostics GmbH., Mannheim Germany, cat. no. 11731297; reference range 11.0-19.5 pmol/l) were measured by electrochemiluminescence immunoassay using a Modular Analytics immunoassay analyzer. The inter-assay coefficients of variation (CVs) were < 5 %.

Plasma total cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos 11876023 and 11875540, respectively; Roche Diagnostics GmBH, Mannheim, Germany). HDL cholesterol was measured with a homogeneous enzymatic colorimetric test (Roche/Hitachi, cat no 04713214; Roche Diagnostics GmbH, Mannheim, Germany). LDL cholesterol was calculated by the Friedewald formula in case of plasma triglycerides <4.5 mmol/l. ApoA-I was assayed by immunoturbidimetry (Roche/Cobas Integra Tina-quant catalog no. 03032566, Roche Diagnostics GmBH, Mannheim, Germany).

Plasma pre ß-HDL was measured by crossed immuno-electrophoresis as described [16,20]. In brief, plasma samples were thawed while kept on ice. 0.9 µmol/L Pefabloc SC (Boehringer-Roche, Penzberg, Germany) and 1.8 µg/l Trasylol (Bayer, Mijdrecht, The Netherlands) were added to inhibit proteolysis (both final concentrations). The crossed immuno-electrophoresis consisted of agarose electrophoresis in the first dimension for separation of lipoproteins with pre β- and α-mobility. Antigen migration from the first agarose gel into the second agarose gel, containing goat anti-human apo A-I antiserum, was used to quantitatively precipitate apo A-I. The antiserum was monospecific for human apo A-I using an immunodiffusion assay. Lipoprotein electrophoresis was carried out in 1% (weight/vol) agarose gels in Tris (80 mmol/l)-tricine (24 mmol/l) buffer, 5% (vol/vol) polyethylene glycol 300 (pH 8.6) and run in an LKB 2117 system (4˚C for 3 h, 210 V). Plasma was applied at 3 µl/well. The track of the first agarose gel was excised and annealed with melted agarose to a gel containing 0.66% (v/v) goat anti-human apo A-I anti-serum (Midland Bioproducts corporation, Boone Iowa) and 0.01% Tween 20 (w/v), that was cast on GelBond film (Amersham, Uppsala, Sweden). The plate was run in an LKB 2117 system (4˚C for 20 h, 50 V) in Tris-tricine buffer. Unreacted antibody was removed by extensive washing saline. The gel was stained with Coomassie Brilliant Blue R250, dried, and scanned with a HP scanjet 5470c. Areas under the pre β-HDL and α-HDL peaks were calculated. The pre ß-HDL area was expressed as the percentage of the sum of apo A-I in the pre β-HDL and the α-HDL areas. Plasma pre ß-HDL formation, i.e. the ability of plasma to generate pre ß-HDL, was determined using the same procedure but now after 24 h incubation of plasma at 37 ^{0}C under conditions of lecithin:cholesterol acyltransferase (LCAT) inhibition [20]. To this end iodoacetate (final concentration 1.0 mmol/l) was added directly after thawing the plasma samples. Pre ß-HDL and pre ß-HDL formation were calculated using the total plasma apo A-I concentration (expressed in apoAI (g/l), and alternatively in % of total plasma apoA-I. The inter-assay CVs were <9 %.

Plasma PLTP activity was assayed with a phospholipid vesicles-HDL system, using [^{14}C]-labeled dipalmitoyl phosphatidylcholine as described [20]. Briefly, plasma samples (1 µl) were incubated with [^{14}C]-phosphatidylcholine-labeled phosphatidylcholine vesicles and excess pooled normal HDL for 45 min at 37 ^{0}C. The method is specific for PLTP activity. Plasma PLTP activity levels vary linearly with the amount of plasma added to the incubation system. PLTP activity was related to the activity in human reference pool plasma and was expressed in arbitrary units (AU; 100 AU corresponds to 13.6 µmol phosphatidylcholine transferred per mL per h). The inter-assay CV of PLTP activity was 5 %.

Statistical analysis

SPSS version 22.0 was used for data analysis. Data are expressed as means ± SD, medians (interquartile ranges) or in numbers. Differences between subjects with and without T2DM were determined by unpaired *t*-tests or Chi-square tests where appropriate. Plasma

triglycerides were not parametrically distributed, and were logarithmically transformed for analysis. Univariate relationships were calculated using Pearson correlation coefficients.

Multivariable linear regression analyses were performed to disclose the independent relationships of plasma pre β-HDL formation and (apo)lipoproteins with thyroid function parameters. In addition, multivariable linear regression analyses were carried out to determine interactions of diabetes status with thyroid function parameters impacting on pre β-HDL formation. Interaction terms were calculated as the product terms of TSH with the presence of T2DM or with HbA1c. To account for possible outliers the distributions of continuous variables were centered to their mean value by subtracting the individual value from the group mean value. Interaction terms were considered to be statistically significant at two-sided *P*-values <0.10 [21]. Otherwise, the level of significance was set at two-sided *P*-values <0.05.

Results

Of 170 potentially eligible subjects, 16 subjects were excluded based on either a TSH or a free T4 level outside the reference range. As a result, 72 T2DM subjects and 82 non-diabetic control subjects participated in the study (Table 1). In T2DM subjects, median diabetes duration was 5.4 years. All T2DM subjects had been given dietary advice. Nineteen T2DM patients used metformin and 19 patients used sulfonylurea alone. Both drugs were used by 24 patients. Other glucose lowering drugs were not used. Anti-hypertensive medication (mostly angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists and diuretics, alone or in combination) were used by 30 T2DM subjects. None of the non-diabetic subjects used anti-hypertensive drugs (*P*<0.001). Three non-diabetic women used estrogens. T2DM subjects were older, but sex distribution was not different between the groups (Table 1). Blood pressure, BMI, plasma glucose and HbA1c were also higher in T2DM subjects. TSH was similar in T2DM and non-diabetic subjects, but free T4 levels were slightly in T2DM subjects (Table 1). This difference was not significant after adjustment for age, sex and the use of glucose lowering drugs (*P*=0.066).

Table 1. Clinical characteristics, thyroid function parameters, phoshoplipid transfer protein (PLTP) activity, plasma lipids, high density lipoprotein (HDL) cholesterol, apolipoprotein A-I (apoA-I), HDL characteristics, pre β-HDL formation, and in subjects with 72 Type 2 diabetes mellitus (T2DM) and in 82 non-diabetic subjects.

	T2DM subjects (n=72)	**Non-diabetic subjects** (n=82)	*P*-value
Age (years)	59 ± 9	55 ± 10	0.029
Sex (men/women)	47/25	47/35	0.31
Systolic blood pressure (mm Hg)	143 ± 20	131 ± 19	<0.001
Diastolic blood pressure (mm Hg)	87 ± 9	82 ± 11	0.009
BMI (kg/m^2)	28.4 ± 4.6	26.0 ± 3.8	0.001
Plasma glucose (mmol/l)	9.0 ± 2.3	5.7 ± 0.7	<0.001
HbA1c (mmol/mol)	51 ± 8	40 ± 3	<0.001
TSH (mU/l)	1.54 ± 0.75	1.65 ± 0.60	0.33
Free T4 (pmol/l)	14.10 ± 1.50	13.58 ± 1.41	0.030
PLTP activity (AU)	103.3 ± 11.7	93.6 ± 10.2	<0.001
Total cholesterol (mmol/l)	5.41 ± 0.91	5.72 ± 0.96	0.037
Triglycerides (mmol/l)	1.78 (1.17-2.47)	1.27 (0.89-1.92)	0.039
LDL cholesterol (mmol/l)	3.30 ± 0.78	3.53 ± 0.86	0.094
HDL cholesterol (mmol/l)	1.24 ± 0.35	1.49 ± 0.41	<0.001
ApoA-I (g/l)	1.34 ± 0.22	1.43 ± 0.22	0.01
HDL cholesterol/apoA-I ratio (mmol/g)	0.91 ± 0.13	1.03 ± 0.17	<0.001
Pre β-HDL (in apoA-I, g/l)	0.055 ± 0.020	0.051 ± 0.019	0.28
Pre β-HDL (in % of apoA-I)	4.16 ± 1.58	3.63 ± 1.24	0.022
Pre β-HDL formation (in apoA-I, g/l)	0.30 ± 0.06	0.31 ± 0.07	0.22
Pre β-HDL formation (in % of apoA-I)	22.5 ± 4.5	21.9 ±4.4	0.44

Data are means ± SD and medians (interquartile ranges) and numbers. Low density lipoprotein (LDL) cholesterol was calculated in 68 T2DM subjects and in 80 non-diabetic subjects. BMI: body mass index; HbA1c: glycated hemoglobin.

Plasma PLTP activity and triglycerides were elevated, whereas total cholesterol was lower in T2DM (Table 1). Non-HDL cholesterol and LDL cholesterol were not significantly different between T2DM and non-diabetic subjects. HDL cholesterol, apoA-I and the HDL cholesterol/apoA-I ratio was decreased in T2DM. Plasma pre β-HDL (expressed in apoA-I concentration) was not different between the groups, but the relative amount of pre β-HDL (expressed in % of apoA-I) was higher in T2DM (Table 1). Pre β-HDL formation (both expressed in apoA-I concentration and in % of apoA-I) was not different between the groups. Pre β-HDL and pre β-HDL formation (expressed in % of apoA-I) were higher in men than in women (4.20 ± 1.44 vs. 3.37 ± 1.26 %, $P<0.001$ and 22.9 ± 4.7 vs. 21.03 ± 3.68, $P=0.007$, respectively).

Pre β-HDL formation (expressed in apoA-I concentration) was correlated positively with pre β-HDL determined without incubation of plasma under conditions of LCAT inhibition in T2DM subjects, non-diabetic subjects and in all subjects combined (Table 2). Pre β-HDL formation was correlated positively with PLTP activity in T2DM subjects and in all subjects combined, whereas this relationship was close to significance in non-diabetic subjects ($P=0.063$). Pre β-HDL and pre β-HDL formation were also correlated positively with plasma total cholesterol and triglycerides, except for a non-significant relation of pre β-HDL formation with triglycerides in non-diabetic subjects (Table 2).

Table 2. Univariate correlations of plasma pre β-HDL and pre β-HDL formation with plasma phospholipid transfer protein (PLTP) activity, total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, apolipoprotein A-I (apoA-I) and the HDL cholesterol/apoA-I ratio in 72 subjects with Type 2 diabetes mellitus (T2DM) and 82 in non-diabetic subjects.

	T2DM subjects (n=72)		**Non-diabetic subjects** (n=82)		**All subjects combined** (n=154)	
	Pre β-HDL (in apoA-I)	Pre β-HDL formation (in apoA-I)	Pre β-HDL (in apoA-I)	Pre β-HDL formation (in apoA-I)	Pre β-HDL (in apoA-I)	Pre β-HDL formation (in apoA-I)
Pre β-HDL		0.247*		0.415***		0.325***
PLTP activity	0.063	0.359**	0.045	0.208	0.085	0.215**
Total cholesterol	0.280*	0.286*	0.370***	0.555***	0.307***	0.445***
Triglycerides	0.298*	0.359**	0.285**	0.123	0.301***	0.078

*Pearson correlation coefficients are shown. Triglycerides are logarithmically transformed. HDL: high density lipoproteins. *P < 0.05; **P < 0.01; ***P ≤ 0.001.*

In T2DM subjects, total cholesterol and triglycerides were correlated positively with TSH, whereas triglycerides were also correlated inversely with free T4 in univariate analysis (Table 3). PLTP activity, HDL cholesterol, apoA-I and the HDL cholesterol/apoAI ratio were not significantly correlated with TSH or with free T4. As shown in Fig. 1, pre β-HDL formation (both expressed in apoA-I concentration and in % of apoA-I) was correlated positively with free T4 in T2DM. Neither in non-diabetic subjects, nor in all subjects combined, significant univariate relationships of these (apo)lipoprotein variables with TSH or with free T4 were observed (Fig. 1), except for a trend of a positive correlation of apoA-I with TSH in non-diabetic subjects (*P*=0.058) (Table 3).

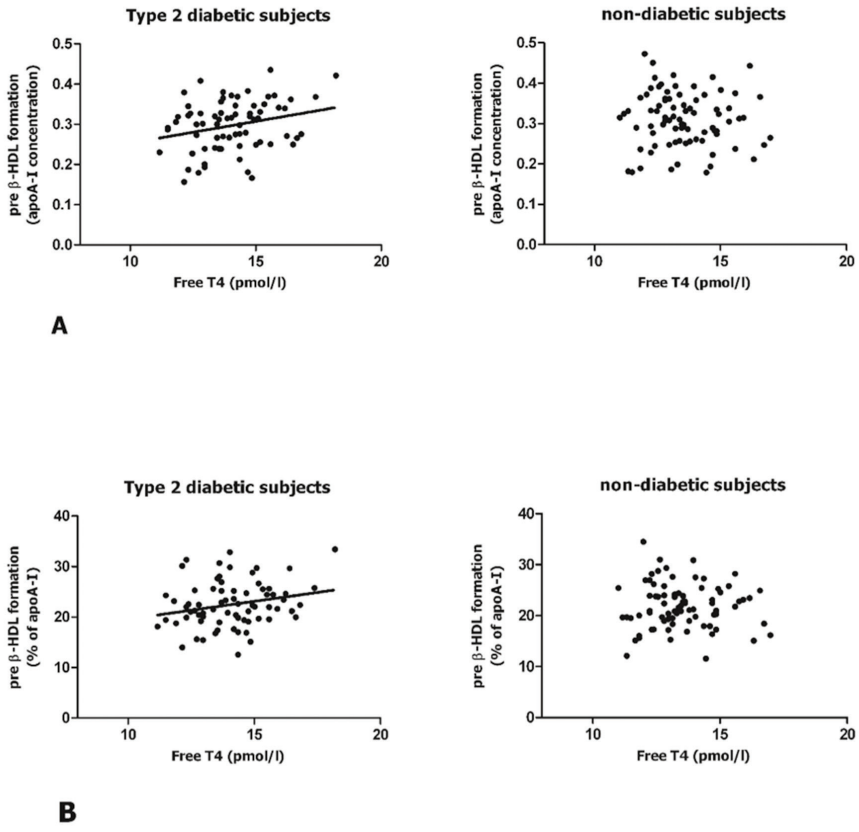
Multivariable linear regression analyses were carried out to determine the independent relationship of pre β-HDL formation with free T4 in T2DM and non-diabetic subjects separately. In age- and sex-adjusted analyses, we included PLTP activity, total cholesterol and triglycerides, representing variables with which pre β-HDL formation was correlated in univariate analysis (Table 2). In T2DM subjects, pre β-HDL formation (expressed in apoA-I concentration) was related positively to free T4, independent of PLTP activity, total cholesterol and triglycerides (Table 4, model 1). Pre β-HDL formation (expressed in % of apoA-I) was also independently related to free T4 (Table 4, model 2). The relationship of pre β-HDL formation with free T4 was essentially unaltered after additional adjustment for the use of metformin, sulfonylurea and anti-hypertensive medication (cf. Table 4, model 1: β=0.237, *P*=0.051; model 2: β=0.333, *P*=0.007; data not shown). In contrast, pre β-HDL formation (both expressed in apoA-I concentration and in % of apoA-I) was not significantly related to free T4 in non-diabetic subjects (Table 4, models 1 and 2). Indeed, the relationship of pre β-HDL formation was found to be modified in the context of chronic hyperglycemia, as indicated by the significant interaction between the presence of T2DM and free T4 impacting on pre β-HDL formation (interaction term with pre β-HDL expressed in apoA-I concentration: *P*=0.022 and in % of apoA-I: *P*=0.010, respectively).

Table 3. Univariate correlations of thyroid function parameters with plasma phospholipid transfer protein (PLTP) activity, total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, apolipoprotein A-I (apoA-I), HDL cholesterol/apoA-I ratio, pre β-HDL and pre β-HDL formation in 72 subjects with Type 2 diabetes mellitus (T2DM) and 82 non-diabetic subjects.

	T2DM subjects (n=72)		**Non-diabetic subjects** (n=82)		**All subjects combined** (n=152)	
	TSH	Free T4	TSH	Free T4	TSH	Free T_4
PLTP activity	0.101	-0.052	-0.096	-0.005	-0.020	0.045
Total cholesterol	0.260*	-0.075	0.098	0.027	0.189	-0.050
Triglycerides	0.276*	-0.247*	-0.008	0.086	0.138	-0.051
HDL cholesterol	-0.088	0.095	0.135	-0.061	0.045	-0.047
ApoA-I	-0.104	0.039	0.210	-0.062	0.063	-0.049
HDL cholesterol/apoA-I ratio	-0.108	0.167	0.027	-0.074	-0.009	-0.036
Pre β-HDL (in apoA-I, g/l)	0.073	0.158	0.002	-0.075	0.033	0.056
Pre β-HDL formation (in apoA-I, g/l)	-0.055	0.260*	0.141	-0.045	0.050	0.077
Pre β-HDL (in % of apoA-I)	0.129	0.119	-0.105	-0.030	0.015	0.081
Pre β-HDL formation (in % of apoA-I)	0.015	0.233*	-0.007	-0.012	-0.001	0.119

*Pearson correlation coefficients are shown. Triglycerides are logarithmically transformed. LDL: low density lipoproteins; HDL: high density lipoproteins; LDL cholesterol was calculated in 68 T2DM subjects and in 80 non-diabetic subjects. *P < 0.05.*

Figure 1.



A. Relationship of pre β-HDL formation (expressed in apoA-I concentration) with free T4 in 72 Type 2 diabetic subjects (left panel) and in 82 non-diabetic subjects (right panel).

B. Relationship of pre β-HDL formation (expressed in % of apoAI) with free T4 in 72 Type 2 diabetic subjects (left panel) and in 82 non-diabetic subjects (right panel).

Table 4. Multivariable linear regression analyses demonstrating relationships of plasma pre β-HDL formation with free T4 in 72 subjects with Type 2 diabetes mellitus (T2DM) and in 82 non-diabetic subjects separately.

	T2DM subjects Model 1 Pre β HDL formation in apoA-I		**T2DM subjects Model 2** Pre β HDL formation in % of apoA-I		**Non-diabetic subjects Model 1** Pre β HDL formation in apoA-I		**Non-diabetic subjects Model 2** Pre β HDL formation in % of apoA-I	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
Age	-0.015	0.89	-0.047	0.67	0.168	0.093	0.033	0.73
Sex (men/ women)	-0.081	0.49	0.207	0.084	-0.051	0.61	0.211	0.035
PLTP activity	0.303	0.014	0.207	0.093	0.140	0.159	0.171	0.081
Total cholesterol	0.255	0.038	0.031	0.80	0.578	<0.001	0.330	0.001
Triglycerides	-0.059	0.64	0.315	0.017	-0.113	0.30	0.262	0.016
Free T4	0.278	0.014	0.343	0.003	-0.096	0.32	-0.047	0.62

β: standardized regression coefficient. Plasma triglycerides are logarithmically transformed. PLTP: phospholipid transfer protein.
Model 1: *Pre β-HDL formation expressed in apoA-I concentration*
Model 2: *Pre β-HDL formation expressed in % of apoA-I*

Discussion

This study has documented a positive univariate relationship of free T4 with plasma pre β-HDL formation in T2DM subjects. This relationship was similarly present when pre β-HDL formation was expressed in plasma apoA-I concentration or in percentage of plasma apoA-I. In contrast, no such relationship was observed in non-diabetic subjects. The diabetic state was indeed found to modify the relationship of pre β-HDL formation with free T4. Furthermore, this relationship remained present taking account of plasma PLTP activity, total cholesterol and triglycerides. It is, therefore, unlikely that the relationship of pre β-HDL formation with free T4 is to a considerable extent explained by diabetes-associated differences in these variables. Collectively, our present results are consistent with the concept that variations in thyroid function within euthyroid range may influence pre β-HDL formation in the context of chronic hyperglycemia.

In the current study population, which included only strictly euthyroid subjects, TSH levels were not different between T2DM and non-diabetic subjects. Free T4 was slightly higher in T2DM subjects, consistent with a recent report [18]. The difference was only 4 %, questioning the pathophysiological relevance of this finding. Moreover, this difference was no longer significant after adjustment for age, sex and the use of glucose lowering medication. In other studies, free T4 was found to be unchanged in T2DM [19,22]. Besides expectedly lower HDL cholesterol and apoA-I levels [22,23], we also noted that the HDL cholesterol/apoA-I ratio was lower in T2DM, which points to a decrease in HDL particle size [22,23,24].

We measured plasma pre β-HDL and pre β-HDL formation using crossed immuno-electrophoresis [20]. The pre β-HDL levels reported here are closely comparable to those reported previously using different analytical methods [14,15,25], but higher absolute plasma pre β-HDL levels have been demonstrated in other reports [12,26]. Although the reasons for these discrepancies are incompletely understood, it is reassuring that both the increase in pre β-HDL concentration in T2DM subjects when expressed in percentage of plasma apoA-I [15,26] and the absolute and relative pre β-HDL formation levels as presently found in T2DM and non-diabetic subjects [20] concur with results from other study populations.

The metabolism of pre β-HDL particles is a complex way governed by a number of factors, such as LCAT lipid transfer proteins and lipases, as well as by the constellation of plasma lipoproteins [9,10,23]. ApoA-I synthesis and the transfer of cell-derived free cholesterol contribute to the generation pre β-HDL particles in the extracellular compartment. Subsequent esterification of free cholesterol in pre β-HDL particles by LCAT plays a key role in the generation of mature, spherical α-HDL [9,10,23]. Pre β-HDL levels thus decrease consequent to LCAT action [27,28], which underlined our approach to additionally measure pre β-HDL after incubation of plasma under conditions of LCAT

inhibition *in vitro* [20]. The metabolism of pre β-HDL is to an important extent also affected by PLTP, which transfers phospholipids to HDL during lipolysis of triglyceride-rich lipoproteins and is able to convert mature, spherical α-HDL into smaller and larger HDL particles [9,10,23]. Therefore, pre β-HDL levels increase as a result of PLTP action. In keeping with other data, we found that pre β-HDL (formation) was related positively to PLTP activity, total cholesterol and triglycerides [20,25], and that plasma PLTP activity was elevated in T2DM [9,23]. The current study also extends recent findings with respect to a positive relationship between low-normal thyroid function and plasma triglycerides, which are ascribed at least in part to increased concentrations of large very low density lipoprotein particles [19,22]. However, plasma PLTP activity was unrelated to variations in thyroid function in the present study. Thus, yet to be more precisely delineated processes could play a role in the relationship of plasma pre β-HDL formation with variation in thyroid function in T2DM.

Assuming that increased plasma pre β-HDL (formation) levels reflect impaired HDL-mediated reverse cholesterol transport [17], it is plausible to postulate that higher pre β-HDL, as presently observed in T2DM, may represent a biomarker of increased atherosclerosis susceptibility [15]. In this vein, it could also be hypothesized that low-normal thyroid function could modify processes making part of the reverse cholesterol transport pathway in T2DM. Further study is required to delineate the possible contribution of the currently observed thyroid function status- pre β-HDL (formation) relationship on the development of atherosclerotic manifestations in the context of chronic hyperglycemia.

Several other methodological considerations and limitations of our study need to be described. First, we performed out a cross-sectional study. Thus, cause-effect relationships cannot be established with certainty. Second, we only measured free T4. However, thyroid hormone effects HDL cholesterol and apoA-I during reversal of hypo- and hyperthyroidism to euthyroidism appear to be sufficiently documented by free T4 measurement alone [30]. Furthermore, variations in free T4 in the context of differences in TSH within the euthyroid range are more outspoken than variations in free T3 [31]. It, therefore, seems unlikely, that free T3 measurement would have provided major additional information regarding the possible association between variation of thyroid function within the euthyroid range and plasma pre β-HDL. Third, subjects using lipid lowering medication were excluded from the present study. Therefore, T2DM subjects with relatively modest changes in plasma lipoproteins preferentially participated. This selection criterion is relevant because statin treatment decreases pre β-HDL (formation) [32]. Fourth, metformin treatment could influence pituitary-thyroid hormone feedback regulation [33], although no independent effect of metformin therapy on the TSH level was found in T2DM subjects [34]. In the present study, the relationship of pre β-HDL with free T4 in T2DM was essentially unaltered taking account of the use of metformin.

In conclusion, this study shows that variation in free T4 within the euthyroid range may affect HDL metabolism by affecting pre β-HDL formation in T2DM.

Conflict of interest

This study is investigator driven. The authors state no conflict of interest.

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6.

Serum paraoxonase-1 activity is inversely related to free thyroxine in euthyroid subjects: The PREVEND Cohort Study

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Abstract

Background: Low-normal thyroid function within the euthyroid range has been suggested to enhance atherosclerosis susceptibility. Paraoxonase-1 (PON-1), may protect against atherosclerotic cardiovascular disease development by attenuating oxidative stress. We evaluated relationships of PON-1 with TSH, free T_4, free T_3, lipids and apolipoprotein (apo) A-I in euthyroid subjects, and assessed whether such relationships are modified in the context of the metabolic syndrome (MetS).

Materials and Methods: Serum PON-1 activity (arylesterase activity), TSH, free T_4, free T_3, lipids and apoA-I were measured in 2206 euthyroid subjects (aged 28 to75 years; 1138 men (age 49 ± 13 years) and 1068 women (age 46 ± 12 years), recruited from the general population (PREVEND cohort).

Results: In age- and sex-adjusted analysis, PON-1 activity (divided into tertiles) was positively related to TSH (β=-0.045, *P*=0.036) and inversely to free T_4 (β=-0.042, *P*=0.050), but not to free T_3 (β=-0.027, *P*=0.20). PON-1 activity was positively related to total cholesterol, non-HDL cholesterol and triglycerides, as well as to HDL cholesterol and apoA-I (*P*<0.01 to <0.001). The inverse relationship of PON-1 activity with free T_4 remained present after adjustment for lipids and other potential confounders (β=-0.066, *P*=0.002), but the positive relationship with TSH lost significance (β=0.034, *P*=0.11). The inverse relationship of PON-1 activity with free T_4 was not different in subjects with vs. without MetS (*P*=0.94), nor modified by the presence of its individual components (*P*≥0.22 for each).

Conclusions: Serum PON-1 activity is inversely associated with free T_4 in euthyroid subjects, suggesting that low-normal thyroid function may affect PON-1 regulation.

Introduction

Low-normal thyroid function, as indicated by a higher thyroid stimulating hormone (TSH) or lower thyroid hormone levels within the euthyroid reference range, may contribute to the development of atherosclerotic cardiovascular disease (CVD) [1-4]. The mechanisms responsible for the association of (subclinical) atherosclerosis with low-normal thyroid function are still incompletely understood. Low-normal thyroid function is associated with a modest increase in plasma total cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides [4-7]. Low-normal thyroid function may also attenuate high density lipoproteins (HDL) function, such as its ability to protect against oxidative stress [4,8].

Accumulating evidence supports the hypothesis that systemic oxidative stress, as at least in part reflected by enhanced oxidative modification of LDL, may contribute to the development of atherosclerosis [9-11]. In this context, it is relevant that LDL oxidation *in vitro* is exaggerated in both hypothyroidism and hyperthyroidism [12,13]. Moreover, increased circulating oxidized LDL levels have been demonstrated in euthyroid subjects with higher TSH levels [14]. Paraoxonase-1 (PON-1) is a HDL-associated hydrolytic enzyme with important anti-oxidative properties [15]. PON-1 hydrolyzes lipid peroxides, thereby preventing their accumulation in LDL particles [15,16]. Studies in rodent models and humans have suggested that the anti-atherogenic effects of the HDL fraction are to a considerable extent attributable to PON-1 activity [16]. PON-1 activity has been shown to be impaired in patients with metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM) and hypercholesterolemia [15,17-19]. Furthermore, lower serum PON-1 activity may predict increased risk of coronary events [20-21] , although the association of PON-1 activity with increased CVD risk was not independent of HDL cholesterol [22].

The effect of thyroid dysfunction on serum PON-1 activity has only been determined in a limited number of studies [23-27]. Remarkably, PON-1 activity was found to be impaired in both hypothyroidism and hyperthyroidism [23]. In addition, PON-1 activity was decreased in (subclinical) hypothyroidism in some [24, 25], but not in other studies [26-28]. No data are currently available concerning the association of serum PON-1 activity in the context of variations in thyroid hormone levels within the euthyroid range.

Against this background, we performed the present study to evaluate the relationships of serum PON-1 activity with thyroid function in euthyroid subjects. In view of decreased PON-1 activity in MetS [15] and potential alterations in thyroid hormones in MetS [4], we also determined the extent to which such a relationship is modified by the presence of MetS and its individual components.

Subjects and Methods

2.1 Subjects

Reporting of the study conforms to STROBE (STrengthening the Reporting of OBservational studies in Epidemiology) statement along with references to STROBE statement and the broader EQUATOR guidelines [29].

The study population consisted of a random subset of participants of the PREVEND (Prevention of Renal and Vascular End Stage Disease) cohort, aged 28–75 years, living in the city of Groningen, The Netherlands. Participants were predominantly of Caucasian origin (94.2%). The protocol of this study has been described in detail elsewhere [30,31]. The local medical ethical committee approved the study; all participants gave written informed consent. For the current analysis, we excluded subjects not being euthyroid, subjects using thyroid hormones, anti-thyroid drugs, amiodarone and lithium carbonate. Euthyroidism was defined as TSH, free T_4 and free T_3 levels each within the respective reference range as provided by the manufacturer (see Laboratory Analyses). We additionally excluded subjects with positive anti-thyroid peroxidase autoantibodies (cut-off value: see Laboratory Analyses). Information on self-reported medication use was combined with information from a pharmacy-dispensing registry, which has complete information on drug of >95% of subjects in the PREVEND study. Applying these selection criteria 2206 subjects were eligible for the current analyses. The presence of a self-reported history of myocardial infarction, percutaneous coronary intervention, coronary artery bypass surgery, stroke or the diagnosis of narrowing of one or both carotid arteries was defined as CVD. Type 2 diabetes mellitus (T2DM) was defined as a fasting serum glucose concentration >7.0 mmol/L, a nonfasting plasma glucose concentration >11.1 mmol/L, a self-report of a physician diagnosis, or the use of glucose-lowering drugs. In order to categorize subjects with the metabolic syndrome (MetS) 3 or more of the following criteria were required: waist circumference > 102 cm for men and > 88 cm for women, hypertension (blood pressure ≥ 130⁄85 mmHg or use of anti-hypertensive drugs), fasting plasma triglycerides ≥ 1.70 mmol/L, fasting glucose ≥ 5.6 mmol/L (or use of glucose lowering drugs), and HDL cholesterol < 1.03 mmol/L for men and < 1.29 mmol/L for women applying NCEP ATPIII criteria [32].

Patient characteristics including age, sex, alcohol use, smoking status, body mass index (BMI), waist circumference, systolic and diastolic blood pressure were obtained. The participants were instructed to let venous blood samples being drawn after an overnight fast for measurement of, glucose, total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, TSH, free T_4 and free T_3 and PON-1 activity. Urinary albumin excretion (UAE) was documented as the mean of two 24-hour urine collections. Body mass index (BMI) was defined as weight (kg) by height (m) squared. Waist circumference (WC) was measured on bare skin between the 10th rib and iliac crest. Alcohol consumption

was recorded with one drink being assumed to contain 10 grams of alcohol. Smoking was categorized into current, former and never. Estimated glomerular filtration rate (eGFR) was calculated with the use of the combined creatinine-cystatin C-based Chronic Kidney Disease Epidemiology Collaboration equation [33].

2.2 Laboratory analyses

Heparinized plasma samples were stored at -80ºC until analyses. Sera were stored at -80ºC until analyses. Serum TSH (Architect; Abbott Laboratories, Abbott Park, IL, USA; reference range 0.35 - 4.94 mU/L), free T_4 (AxSYM; Abbott Laboratories, Abbott Park, IL, USA; reference range 9.14 – 23.81 pmol/L) and free T_3 (AxSYM; Abbott Laboratories, Abbott Park, IL, USA; reference range; 2.23 - 5.35 pmol/L) were measured by microparticle enzyme immunoassays. Anti-thyroid peroxidase autoantibodies were determined using commercially available automated enzyme linked immunoassays (Abbott Laboratories, Abbott Park, IL, USA; kit number 5F57). Anti-thyroid peroxidase autoantibodies were considered positive using a cut-off value as indicated by the supplier (≥12 kU/L).

Serum PON-1 enzymatic activity was measured as its arylesterase activity, i.e. as the rate of hydrolysis of phenyl acetate into phenol, as described [34]. The inter-assay CV was 8%. Arylesterase activity, measured with this assay, is positively correlated with PON-1 enzymatic activity toward paraoxon as well as with PON-1 mass [35].

Total serum cholesterol and plasma glucose were measured using Kodak Ektachem dry chemistry (Eastman Kodak, Rochester, NY, USA). Serum triglycerides were measured enzymatically. HDL cholesterol was measured with a homogeneous method (direct HDL, AEROSET system; Abbott Laboratories, Abbott Park, IL, USA; no. 7D67). Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. Serum apoA-I was determined by nephelometry applying commercially available reagents for Dade Behring nephelometer systems (BN II; Dade Behring, Marburg, Germany; apoA-I test kit, code no. OUED).

Serum creatinine was measured by an enzymatic method on a RocheModular analyzer (Roche Diagnostics, Mannheim, Germany). Serum cystatin C was measured by Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a Modular analyzer (Roche Diagnostics). Urinary albumin concentration was measured by nephelometry with a threshold of 2.3 mg/l (Dade Behring Diagnostic, Marburg, Germany).

2.3 Statistical analyses

Data analysis was performed using IBM SPSS software (version 23.0, SPSS Inc. Chicago, IL, USA). Normally distributed data are given as mean ± SD and non-parametrically distributed data are presented as median (interquartile range). Categorical variables are given as percentages. Differences in PON-1 activity between men and women were determined by Mann-Whitney U-test. Clinical and laboratory characteristics of the study

population are presented according to sex-stratified tertiles of PON-1 activity. Differences in proportions of dichotomous variables across tertiles of PON-1 activity were determined by multinomial χ-square tests. Multivariable linear regression analyses, adjusted for age and sex, were used to test for linear trends between tertiles of PON-1 activity. Age- and sex-adjusted multivariable linear regression analyses were also used to determine the extent to which PON-1 activity (as continuous variable) was related to thyroid function parameters (TSH, free T_4, free T_3) taking clinical and laboratory covariates into account. PON-1 activity, TSH, triglycerides and UAE were natural logarithm (log_e) transformed in order to achieve approximately normal distributions. Interaction terms were calculated as the product term of TSH or free T_4 with sex or the presence of MetS or its component of interest. To account for outliers the individual TSH or free T_4 values were centered to the mean by subtracting the group mean value from individual values [36,37]. Interaction terms were considered statistically significant at *P*-values <0.10, as recommended by Selvin [38]. Otherwise, two-sided *P*-values <0.05 were considered significant.

Results

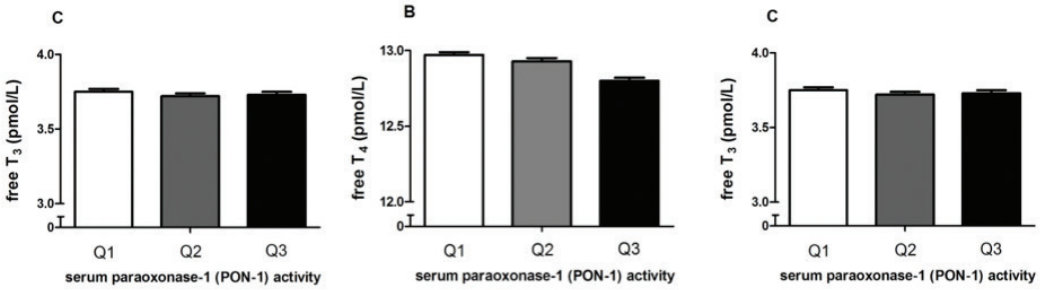
Mean age of the 2206 participants was 48 ± 13 years. 1138 participants (52.6 %) were men (age 49 ± 13 years) and 1068 (47.4 %) were women (age 46 ± 12 years). Serum PON-1 activity amounted to 56.1 (46.1 - 68.1) U/L in the whole population, and was 60.0 (46.7 - 70.5) U/L in women vs. 54.9 (45.8 - 66.0) U/L in men (*P*=0.002). Clinical and laboratory characteristics of the study population are, therefore, shown according to sex-stratified tertiles of PON-1 activity (Table 1). One hundred two participants (4.6 %) reported a previous cardiovascular event, 65 subjects (2.9 %) had T2DM and 418 (19%) subjects fulfilled the criteria for MetS. A history of CVD (*P*= 0.002) and the presence of MetS (*P*=0.013) was more prevalent in subjects categorized in the lowest tertile of PON-1 activity, but diabetes status did not significantly vary according to the PON-1 categories (*P*=0.096) (Table 1). Oral glucose lowering drugs were used by 36 subjects, lipid modifying drugs (mainly statins) by 113 participants and antihypertensives by 274 subjects. Oral contraceptives were used by 283 women. The use of oral glucose lowering drugs (*P*<0.002, antihypertensives (*P*<0.001) was more prevalent in the subjects belonging to the lowest tertile of PON-1 activity, whereas the use of oral contraceptives was more prevalent in the women belonging to the highest tertile of PON-1 activity (*P*<0.001) (data not shown). The use of lipid lowering drugs did not vary across tertiles of PON-I activity (*P*=0.17). Accordingly, serum PON-1 activity was lower in subjects using oral glucose lowering drugs (45.9 (39.5-56.8 U/L) vs. 56.2 (46.3-68.4) U/L, *P*<0.001), in subjects using antihypertensives (52.6 (41.8-64.7 U/L) vs. 56.7 (46.8-68.8 U/L), *P*<0.001) and in women using oral contraceptives (62.9 (52.2-76.7 U/L) vs. 55.2 (45.7-66.7 U/L), *P*<0.001), but was not different in subjects using lipid lowering drugs

compared to those who did not (56.5 (46.6-64.2 U/L vs. 56.1 (46.1-67.5) U/L, *P*=0.10). Serum PON-1 activity was inversely related to age. In age- and sex-adjusted analysis, PON-1 activity was positively related to systolic and diastolic blood pressure (Table 1). PON-1 activity was unrelated to BMI, waist circumference, glucose, eGFR and UAE, and did not vary significantly according to smoking status and alcohol consumption. Additionally, PON-1 activity was positively related to total cholesterol, non-HDL cholesterol, triglycerides, as well as to HDL cholesterol and apoA-I (Table 1). Of note, in age- and sex-adjusted analysis, PON-1 activity was positively related to TSH and inversely to free T_4, but not to free T_3 (Table 1). Fig.1 shows TSH, free T_4 and free T_3 levels according to sex-stratified tertiles of PON-1activity. There were no interactions of sex with TSH, free T_4 or free T_3 on PON-1 activity (*P*=0.52 to *P*=0.64; data not shown).

Table 1. Clinical and laboratory characteristics in 2206 subjects according to sex-stratified tertiles of paraoxonase-1 (PON-1) activity.

	Sex stratified tertiles of PON-1 activity (U/L)				
	1 Men 19.7-48.8 Women 17.0-50.2	2 Men 48.9-62.2 Women 50.2-65.1	3 Men 62.2-130.7 Women 65.2-119.3	β	*P*-value
Participants, n	735	736	735		
Men, n (%)	379 (51.6)	380 (51.6)	379 (51.6)		
Women, n (%)	356 (48.4)	356 (48.4)	356 (48.4)		
Age (years)	49.8 ± 13.2	48.1 ± 12.6	45.7 ± 11.4	-0.132	<0.001
BMI (kg/m^2)	25.9 ± 4.3	26.1 ± 4.6	25.4 ± 3.9	-0.013	0.65
Waist circumference (cm)	87.8 ± 13.3	88.1 ± 13.5	86.3 ± 12.7	0.000	0.98
Systolic blood pressure (mmHg)	129 ± 20	129 ± 21	127 ± 18	0.038	0.037
Diastolic blood pressure (mmHg)	74 ± 9	74 ± 10	74 ± 10	0.053	0.004
Alcohol					0.43
< 10 gram per day (%)	538 (73.6)	521 (71.2)	518 (70.8)		
≥ 10 gram per day (%)	193 (26.4)	211 (28.8)	214 (29.2)		
Smoking					0.081
never (%)	188 (25.8)	221 (30.2)	221 (30.1)		
former (%)	246 (33.7)	245 (33.5)	263 (35.8)		
current (%)	296 (40.5)	266 (36.3)	250 (34.1)		
Glucose (mmol/L)	4.6±1.3	4.6±1.1	4.5±1.1	0.007	0.74
Total cholesterol (mmol/L)	5.5 ± 1.14	5.6 ± 1.22	5.7 ± 1.12	0.12	<0.001
Non-HDL cholesterol (mmol/L)	4.2 ± 1.25	4.2 ± 1.31	4.3 ± 1.22	0.069	<0.001
HDL cholesterol (mmol/L)	1.28 ± 0.39	1.36 ± 0.40	1.42 ± 0.42	0.131	<0.001
Triglycerides (mmol/L)	1.14 (0.81-1.63)	1.12 (0.82-1.62)	1.16 (0.85-1.69)	0.057	0.005
ApoA-I (g/L)	1.35 ± 0.32	1.40 ± 0.31	1.44 ± 0.33	0.127	<0.001
ApoB (g/L)	1.04 ± 0.35	1.04 ± 0.34	1.03 ± 0.33	0.034	0.099
CVD (n, %)	46 (6.3)	38 (5.2)	18 (2.4)		0.002
MetS (n, %)	161 (21.9)	140 (19.0)	117 (15.9)		0.013
T2DM (n, %)	29 (3.9)	21 (2.9)	15 (2.0)		0.096
eGFR (ml/min/1.73m^2)	95.1 (83.2-105.9)	97.3 (86.3-107.8)	98.7 (87.5-109.6)	0.020	0.23
UAE (mg/24 hrs)	9.0 (6.1-17.2)	9.0 (6.2-16.9)	8.6 (6.1-15.7)	0.028	0.18
TSH (mU/L)	1.28 (0.94-1.80)	1.29 (0.95-1.81)	1.39 (0.99-1.87)	0.045	0.036
free T_4 (pmol/L)	12.97 ± 1.83	12.93 ± 1.71	12.80 ± 12.8	-0.042	0.050
free T_3 (pmol/L)	3.75 ± 0.64	3.72 ± 0.61	3.73 ± 0.62	-0.027	0.20

Figure 1. TSH, and free T4 and free T3 levels according to sex-stratified tertiles of serum paraoxonase-1 (PON-1) activity. P-values for linear trend (adjusted for age and sex): TSH: P=0.036, free T4: P=0.050 and free T3: P=0.20. Data are given in means and standard errors. TSH is logarithmically transformed.



Data in mean ± SD or in median (interquartile range). For continuous variables *P*-values for linear trend are adjusted for age and sex, except for age which was adjusted for sex only. Data with respect to smoking and alcohol consumption are missing in 10 (0.5%) and 11 (0.5%) of the subjects, respectively. Triglycerides, UAE and TSH are $\log_e$ transformed. For dichotomous variables *P*-values are by multinomial χ-square test. Apo, apolipoprotein; BMI, body mass index; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate, HDL, high density lipoproteins; MetS, metabolic syndrome; T2DM, type 2 diabetes mellitus; UAE, urinary albumin excretion. β: standardized regression coefficient. We then tested whether the relationships of PON-1 activity (as continuous variable) with TSH and free T_4 remained present after adjustment for relevant clinical and laboratory covariates (Table 2). In age- and sex-adjusted multivariable linear regression analysis including free T_4 and TSH together PON-1 activity was positively associated with TSH and inversely with free T_4 (Table 2, model 1). In analysis with free T_4, free T_3 and TSH together, there was no significant independent association of PON-1 activity with free T_3 (β=-0.029, *P*=0.18; data not shown). The inverse relationship of PON-1 activity with free T_4 remained present after additional adjustment for non-HDL cholesterol, HDL cholesterol and triglycerides, although the positive relationship of PON-1 activity with TSH lost significance (Table 2, model 2). Likewise, PON-1 activity was inversely related to free T_4 in an alternative model which included apoA-I instead of HDL cholesterol (β= -0.055, *P*=0.01; data not shown). An inverse relationship of PON-1 activity with free T_4 was also found after additional adjustment for systolic and diastolic blood pressure, UAE, eGFR, alcohol consumption, smoking, a previous history of cardiovascular disease and diabetes status (Table 2, model 3), and finally after further adjustment for oral glucose lowering drugs, lipid lowering medication, antihypertensives and oral contraceptives (Table 2, model 4). The inverse relationship of PON-1 activity with free T_4 was not different in subjects with vs. without MetS (*P*=0.94),

nor modified by the presence of its individual components (low HDL cholesterol: *P*=0.58); elevated triglycerides: *P*=0.96); enlarged waist circumference: *P*=0.57); elevated blood pressure: *P*=0.31; elevated glucose: *P*=0.22).

Table 2. Multiple linear regression models demonstrating the independent association of free T4 and TSH with paraoxonase-1 (PON-1) activity.

	Model 1		*Model 2*		*Model 3*		*Model 4*	
	β	*P-value*	*β*	*P-value*	*β*	*P-value*	*β*	*P-value*
Age (years)	-0.141	<0.001	-0.183	<0.001	-0.160	<0.001	-0.131	<0.001
Sex (men vs. women)	-0.050	0.018	-0.009	0.69	-0.005	0.85	0.029	0.276
free T_4 (pmol/L)	-0.064	0.003	-0.067	<0.001	-0.066	0.002	-0.0642	0.002
TSH (mU/L)	0.051	0.016	0.035	0.091	0.034	0.11	0.033	0.124
non-HDL cholesterol (mmol/L)			0.095	<0.001	0.089	0.001	0.097	<0.001
HDL cholesterol (mmol/L)			0.242	<0.001	0.230	<0.001	0.220	<0.001
Triglycerides (mmol/L)			0.116	<0.001	0.121	<0.001	0.103	<0.001
Systolic blood pressure (mm Hg)					-0.010	0.78	-0.005	0.878
Diastolic blood pressure (mm Hg)					0.013	0.70	0.006	0.870
eGFR (ml/min/1.73m^2)					0.029	0.28	0.030	0.271
UAE (mg/24 hrs)					0.033	0.15	0.028	0.226
CVD history (yes/no)					-0.021	0.34	-0.016	0.496
Diabetes status (yes/no)					-0.031	0.15	0.039	0.222
Alcohol consumption (< vs. ≥ 10 gram per day)					0.025	0.26	0.029	0.190
Smoking (never, former, current)					-0.049	0.024	-0.050	0.023
Glucose lowering drugs							-0.089	0.004
Lipid lowering drugs							0.007	0.766
Antihypertensives							-0.018	0.469
Oral contraceptives							0.092	<0.001

β: standardized regression coefficient. eGFR, estimated glomerular filtration rate; HDL, high density lipoproteins; UAE, urinary albumin excretion. PON-1 activity, TSH, triglycerides and UAE are loge transformed. Alcohol consumption is categorized in per day. Smoking is categorized in never, former and current. Variables included in the models:

Model 1: *age, sex, free T4, TSH*

Model 2: *model 1 plus non-HDL cholesterol, HDL cholesterol and triglycerides*

Model 3: *model 2 plus systolic and diastolic pressure, alcohol consumption, smoking, plus cardiovascular disease (CVD) history and diabetes status.*

Model 4: *model 3 plus glucose lowering drugs, lipid modifying medication, antihypertensives and oral contraceptives.*

Table 3. Multivariable linear regression analyses demonstrating relationships of paraoxonase-1 (PON-1) activity with after exclusion of subjects with a history of cardiovascular disease and type 2 diabetes mellitus (n=2051; model 1) or oral glucose lowering drugs, lipid lowering drugs, oral contraceptives and antihypertensives (n=1596; model 2).

	Model 1		**Model 2**	
	β	*P*-value	β	*P*-value
Age	-0.161	<0.001	-0.140	<0.001
Sex (men vs women)	-0.011	0.67	-0.026	0.37
TSH	0.028	0.21	0.047	0.06
free T_4	-0.047	0.035	-0.049	0.049

β: standardized regression coefficient. All models are adjusted for age, sex, non-high density lipoprotein (HDL) cholesterol, HDL cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, alcohol consumption and smoking. PON-1 activity, TSH and triglycerides are loge transformed.

Discussion

In this large population-based study among strictly euthyroid subjects, we have shown to our knowledge for the first time, that serum PON-1 activity is positively related to TSH and inversely to free T_4 in age- and sex-adjusted analysis. In multivariable logistic regression analysis in which we included TSH, free T_4 and free T_3 together and adjusted for lipoproteins and other potentially important covariates, the inverse association of PON-1 activity with free T_4 remained present. The inverse relationship of PON-1 activity with free T_4 was not different between subjects with and without MetS nor modified by the presence of its individual components. Our current results are, therefore, in agreement with the hypothesis that variations in thyroid function within the euthyroid range may affect serum PON-1 activity.

In the interpretation of the results it is relevant that serum PON-I activity was assayed with phenyl acetate as substrate. Arylesterase activity, as measured with this type of assay, is widely used in large scale studies, and has the advantage of an approximately normal distribution, making it suitable for multivariable modeling [22]. Moreover, PON-I activity towards phenyl acetate is less variable between subjects compared to its activity towards paraoxon [overviewed in 18]. As expected [22,39-41] , PON-1 activity was positively related to HDL cholesterol and apoA-I. Its correlation with non-HDL cholesterol and triglycerides is probably explained by an association of PON-1 with very low density lipoproteins which are able to act as a vector for its cellular secretion [42]. Such relations of PON-1 activity with circulating lipoproteins together with the effect of low-normal thyroid function to increase plasma cholesterol and triglycerides [4,5,43] underscore the necessity to adjust for (apo) lipoprotein levels when evaluating the relationship of PON-1 activity with variation in thyroid function in euthyroid subjects. In the present study we only included euthyroid subjects using strict criteria, i.e. TSH, free T_4 and free T_3 each being within their respective reference range, as done in other reports [44,45]. Moreover, we excluded subjects with positive anti-thyroid peroxidase autoantibodies to avoid possible confounding of latent thyroid autoimmunity on inflammatory and oxidative stress as much as possible [25].

Inconsistent effects of thyroid function status on PON-1 activity have been reported so far [24-28]. The inverse relation of PON-1 activity with free T_4 as shown in the current study suggests that low-normal thyroid function could contribute to higher PON-1 activity, although it should be emphasized that this relationship was modest. The mechanisms responsible for this relationship are not yet known. It is unclear whether thyroid hormones are able to affect PON-1 gene expression. PON-1 is down regulated by interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [46]. Hypothyroidism may increase IL-1 and TNF-α [47], whereas higher levels of TNF-α are also found in subjects with low-normal thyroid function [48]. In addition, oxidized lipids are recognized to inhibit PON-1

activity [49-51]. In this context, it is relevant that low density lipoprotein (LDL) oxidation *in vitro* is exaggerated in both hypothyroidism and hyperthyroidism [12,13]. Moreover, increased levels of oxidized LDL have been demonstrated in euthyroid subjects with high normal TSH levels [14]. Taken together, these data [14,46-48] make it unlikely that a higher PON-1 activity in relation to low normal thyroid function is to explained by thyroid hormone- mediated effects on IL-1 and TNF-α or on (systemic) oxidative stress.

Given the inverse though modest relation of PON-1 activity with free T_4, it seems plausible that other mechanisms than effects of PON-1 on oxidative stress defense could contribute to the previously reported enhanced oxidative stress in the context of low normal thyroid function [14]. It also seems unlikely that changes in PON-1 activity play a major role in an attenuated ability of HDL to protect against LDL oxidation *in vitro* in subjects with low-normal thyroid function [8], a read-out of HDL functionality which is closely related to PON-1 activity [52]. In this regard, is relevant that other factors affecting oxidative stress such as superoxide dismutase [53] and circulating bilirubin levels [44] are also affected by thyroid function. Of note, it has been demonstrated recently that the inverse relationship of bilirubin with free T4 is stronger in more insulin resistant individuals [44], and that the relationship of PON-1 activity with its activator, apoE, is impaired in subjects with MetS [41]. For this reason we also set out to determine whether the relationship of PON-1 activity was modified in the context of MetS. We found that the inverse relationship of PON-1 activity with free T_4 was not modified by the presence of MetS nor by its individual components.

The regulation of PON-1 is dependent on many genetic and environmental factors. Regarding environmental factors, several animal and human studies have shown that dietary lipids can influence PON-1 activity [54-56]. Furthermore it has been reported that physically active subjects have higher PON-1 activity [57]. A limitation of the present study is that detailed information on nutrient intake and data with respect to physical activity were not available. Statins may also increase PON-1 activity [58], although this has not been unequivocally reported [59]. In the current report, PON-1 activity was not affected by the use of lipid lowering drugs. However, PON-1 activity was inversely associated with the use of glucose lowering medication in analysis in which we also adjusted for the presence of T2DM. We explain this finding by assuming that the use of glucose lowering drugs preferentially labels diabetic patients with more severe hyperglycemia, requiring medical drug treatment. Further, PON-1 activity was elevated in women who used oral contraceptives. Although little information is available on this issue, it seems consistent with some other data suggesting that PON-1 activity is higher in women taking oral contraceptives, and may increase in response to ethinyl oestradiol and cyproterone acetate combination [60,61].

Several other methodological aspects and limitations of our study need to be considered. We performed a cross-sectional study, so that conclusions regarding cause-

effect relationships cannot be drawn with certainty. However, we are not aware of any data underscoring a physiological role of PON-1 itself in thyroid hormone regulation. In addition owing to the observational nature of our study, residual confounding due to unmeasured confounders cannot be entirely ruled out. We performed secondary analyses after exclusion of subjects with a history of CVD and T2DM, and the use of glucose lowering, lipid lowering, antihypertensive medication and oral contraceptives. Reassuringly, these analyses showed the same inverse relationship of PON-1 activity with free T_4. Of further note, PON-1 activity was assayed in sera that were stored for a prolonged period. However, loss of PON-1 enzymatic activity are minimal if samples stored frozen at -70 C^0 [62].

In conclusion, this large population-based cohort study demonstrates for the first time that serum PON-1 activity is inversely associated with free T_4 in euthyroid subjects. It is conceivable that low-normal thyroid function may influence PON-1 regulation.

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7.

Tumor necrosis factor-α is inversely related to free thyroxine in euthyroid subjects without diabetes

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Abstract

Lower thyroid functional status within the euthyroid range may confer increased atherosclerosis susceptibility, as evidenced by increased intima media thickness and coronary artery calcification. Associations of lower thyroid functional status with pro-atherogenic (inflammatory) biomarkers may also extend into the euthyroid range. Here we established relationships of plasma tumor necrosis factor-α (TNF-α) with thyroid stimulating hormone (TSH) and free thyroxine (free T_4) in euthyroid subjects with and without Type 2 diabetes mellitus (T2DM). Fasting TSH, free T_4 and TNF-α were measured in 81 non-diabetic subjects and in 73 T2DM subjects with Type 2 diabetes mellitus (T2DM; insulin using subjects were excluded) with TSH and free T_4 levels within their institutional reference ranges. TSH was similar and free T_4 was slightly higher in T2DM (p<0.016). Plasma TNF-α was increased in T2DM (p=0.007). In non-diabetic subjects, TNF-α was correlated inversely with free T_4 (r=-0.254, p=0.022), whereas such a relationship was absent in T2DM subjects (r=0.058, p =0.63). Multivariable linear regression analysis showed that in non-diabetic subjects TNF-α remained inversely associated with free T_4 after adjustment for age and sex (β=-0.243, p=0.032), contrasting the lack of relationship in T2DM subjects (interaction: p=0.053). In T2DM subjects, TNF-α was also unrelated to free T_4 taking account of possible confounders, as well as after exclusion of subjects using metformin or antihypertensive medication. In conclusion, higher levels of TNF-α relate to lower free T_4, suggesting that lower thyroid functional status within the euthyroid range could influence pro-inflammatory pathways. This relationship appears to be disturbed in T2DM.

Introduction

Given that each individual probably has narrow variations in circulating thyroid hormone levels, measurement of a single set of thyroid function parameters is considered to be clinically and pathophysiologically relevant [1-3]. Low-normal thyroid function, as reflected by a higher thyroid stimulating hormone (TSH) or lower thyroid hormone levels within the euthyroid range, could have a negative impact on the development of atherosclerotic cardiovascular disorders [2,3]. In agreement with this concept, low-normal thyroid function associates with increased intima media thickness (cIMT), an established marker of subclinical atherosclerosis [4,5]. Moreover, it was documented recently that low-normal thyroid function is associated with progression of coronary artery calcification [6,7], although a high-normal TSH level is unlikely to predict new onset clinically manifest coronary heart disease [8].

The mechanisms responsible for the association of (subclinical) atherosclerosis with low-normal function are still incompletely understood. Low-normal thyroid function may give rise to small increases in plasma levels of total cholesterol and atherogenic apolipoprotein B-containing lipoproteins [9]. Low-normal thyroid function may also convey changes in high density lipoprotein (HDL) function, which conceivably contribute to impaired oxidative stress defense [9]. Elevated levels of inflammatory markers, such as high sensitivity C-reactive protein (hsCRP), have been linked to the risk of myocardial infarction [10]. Interestingly, several cross-sectional studies have shown that subjects with subclinical hypothyroidism (SCH) have higher serum high sensitivity C-reactive protein (hsCRP) than healthy subjects, although this has not always been reported [4,11-16]. Taken together, it is plausible to postulate that SCH is associated with low-grade chronic inflammation [17].

Tumor necrosis factor alfa (TNF-α) is an established mediator of apoptosis, inflammation and the innate immune system response to different forms of stress, like infection, trauma or ischemia [18,19]. It is well appreciated that TNF-α is important in the development of coronary heart disease and plaque formation [19]. Plasma TNF-α levels are significantly higher in patients with myocardial infarction, whereas patients with persistently higher levels in the post-infarction period are at a threefold higher risk of developing new coronary episodes [20]. Moreover, the plasma TNF-α concentration may correlate positively with cIMT [21]. Plasma TNF-α is increased in a hypothyroid rat model [22]. Higher TNF-α levels in SCH has also been found in humans [23]. In line, expression of TNF-α in macrophages from carotid artery atherosclerotic plaques is enhanced in the context of SCH [24]. It is, therefore, plausible to hypothesize that low-normal thyroid function associates with higher circulating TNF-α levels. However, no data are currently available with respect to the relationship of low-normal thyroid function with TNF-α. Type 2 diabetes mellitus (T2DM) is characterized by low grade chronic inflammation [25].

Exposure to high glucose increases TNF-α release from rat and human aortic smooth muscle cells in culture [26]. Therefore, it is relevant to determine the relationship of low-normal thyroid function with TNF-α in this patient category.

The present study was performed to evaluate the relationships of thyroid stimulating hormone (TSH) and free thyroxine (free T_4) with plasma tumor necrosis factor-α (TNF-α) in euthyroid subjects with and without Type 2 diabetes mellitus (T2DM).

Methods

Study design and Subjects

The study protocol was approved by the medical ethics committee of the University Medical Center Groningen, the Netherlands, and written informed consent was obtained from all participants. Subjects with and without T2DM were aged > 18 years and were recruited by advertisement in local newspapers. Eligible subjects had a serum TSH as well as a serum free T_4 level within the institutional reference range, as outlined below. Current smokers and subjects who used lipid lowering drugs were excluded, as were subjects with a history of cardiovascular disease (CVD), chronic kidney disease (estimated glomerular filtration rate < 60 ml/min/1.73 m^2 or micro/macroalbuminuria), liver disorders (serum transaminase levels >2 times the upper reference limit), thyroid disorders and pregnancy. The use of anti-hypertensive medication and oral contraceptives was allowed. T2DM had been diagnosed previously according to guidelines from the Dutch College of General Practitioners (fasting plasma glucose ≥ 7.0 mmol/l; non-fasting plasma glucose ≥11.1 mmol/l). Diabetic subjects using metformin, sulfonylurea or antihypertensive medication were allowed to participate, but insulin users were excluded.

Physical examination did not reveal pulmonary, cardiac abnormalities or thyroid abnormalities. Body mass index was calculated as weight (kg) divided by height (m) squared. Blood pressure was measured after 15 min of rest at the left arm using a sphygmomanometer. The participants were evaluated between 0800 and 1000 h after an overnight fast. The set-point of pituitary TSH feedback inhibition by free T_4, designated the TSH index (TSHI)), was estimated as follows: TSHI = log TSH + 0.1345 x free T_4 [27,28]. Homeostasis model assessment of insulin resistance ($HOMA_{ir}$) was used to estimate insulin resistance (fasting insulin (mU/l) x fasting glucose (mmol/l)/22.5).

Laboratory measurements

Serum and EDTA-anticoagulated plasma samples, prepared by centrifugation at 1400 g for 15 min at 4^0C, were stored at -80 ^{0}C until analysis. Plasma glucose and glycated hemoglobin (HbA1c) levels were measured shortly after blood collection.

Serum TSH (sandwich principle; Roche Diagnostics GmbH., Mannheim, Germany, cat. no. 117314591; reference range 0.5-4.0 mU/l) and free T_4 (competition principle; Roche

Diagnostics GmbH., Mannheim Germany, cat. no. 11731297; reference range 11.0-19.5 pmol/l) were measured by electrochemiluminescence immunoassay using a Modular Analytics immunoassay analyzer. Anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-Tg) auto-antibodies were measured with enzyme-linked immunoassays (ImmunoCap cat nos. 14-4508-35 and 14-4507-35, respectively; Phadia, Freiburg, Germany), and were considered to be positive using cut-off values provided by the supplier (anti-TPO antibodies > 60 IU/ml and anti-Tg antibodies > 289 IU/ml).

Plasma TNF-α was measured using Luminex xMAP technology (Lincoplex panel B cat. no. HADK1-61K-B; Linco Research Inc., St Charles, MO, USA). Validation experiments have shown that TNF-a levels, as measured with this technology, are strongly correlated (r > 0.80) with assay results obtained by enzyme-linked immunoassays obtained from Linco Inc. (data provided by the manufacturer).

Plasma total cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos 11875540 and 11876023, respectively; Roche Diagnostics GmbH, Mannheim, Germany). HDL cholesterol was measured with a homogeneous enzymatic colorimetric test (Roche/Hitachi, cat no 04713214; Roche Diagnostics GmbH, Mannheim, Germany). Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. Low density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula if the triglyceride concentration was <4.5 mmol/l.

Insulin was measured by microparticle enzyme immunoassay (AxSYM insulin assay: Abbott Laboratories, Abbott Park, IL, USA). The intra-assay coefficients of variation of all assays were less than 6%. HbA1c was measured by high-performance liquid chromatography (Biorad, Veenendaal, the Netherlands, normal range 27-43 mmol/mol).

The intra-assay coefficients of variation of all assays were < 6 %.

Statistical analysis

SPSS 22 (version 22.0, SPSS Inc., Chicago, IL, USA) was used for data analysis. Data are expressed as means ± SD, medians (interquartile ranges) or in numbers. Differences between non-diabetic and T2DM subjects were determined by unpaired T-tests or Chi-square tests. Since TNF-α, insulin, $HOMA_{ir}$ and triglycerides were not parametrically distributed, these variables were logarithmically transformed to compare between group differences, as well as for correlation analyses. Univariate relationships were calculated using Pearson correlation coefficients.

Multivariable linear regression analyses were carried out to disclose the independent relationships of TNF-α with free T_4. We also determined differences in the relationships of TNF-α with free T_4 between diabetic and non-diabetic subjects. To this end the interaction term of free T_4 with diabetes status was calculated as the product of the presence of diabetes (yes/no) with free T_4. This interaction term was considered to be statistically significant at two-sided *p*-values < 0.10 as recommended by Selvin [29]. Otherwise, two-sided *p*-value < 0.05 indicated statistical significance.

Results

Eighty one non-diabetic subjects and 73 T2DM subjects (diabetes duration ranging from 1 to 13 years) were included in the study (Table I). Thyroid auto-antibodies were available in non-diabetic subjects, and were elevated in 6 subjects. Anti-hypertensive medication (in most cases angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists and diuretics, alone or in combination) were used by 30 T2DM subjects and by none of the non-diabetic subjects ($P<0.001$). Fourteen T2DM subjects used metformin and 15 subjects used sulfonylurea alone. Twenty four subjects used both drugs. Other glucose lowering drugs were not used. One pre-menopausal woman and 2 post-menopausal women without diabetes were using estrogens.

Sex distribution was not different between the groups, but the T2DM subjects were somewhat older than non-diabetic subjects. Blood pressure, BMI, plasma glucose and HbA1c were higher in T2DM subjects coinciding higher plasma insulin and $HOMA_{ir}$ values (Table I). TSH was not different between T2DM and non-diabetic subjects, but free T_4 levels were slightly higher in T2DM subjects. TSHI was not different between the groups (Table I). Plasma total cholesterol was lower in T2DM subjects which was mainly due to lower HDL cholesterol. Triglycerides were higher in T2DM subjects. TNF-α levels were also elevated in T2DM subjects (Table I). This difference remained present after adjustment for age and sex ($\beta = 0.201$, $p = 0.014$). In both groups combined, TNF-α was not significantly different between men (3.40 (2.45-4.55) ng/l) and women (3.20 (2.50-3.80) ng/l) ($p = 0.52$).

Table 1. Clinical characteristics, thyroid function parameters, tumor necrosis factor-alpha (TNF-α) in 81 non-diabetic subjects and in subjects with Type 2 diabetes mellitus (T2DM).

	Non-diabetic subjects (n=81)	**T2DM subjects** (n=73)	*p*-value
Age (years)	55 ± 10	58 ± 9	0.036
men	58 ± 10	58 ± 9	0.93
women	52 ± 8	60 ± 10	0.002
Sex (men/women)	46/35	47/26	0.43
Systolic blood pressure (mm Hg)	131 ± 19	143 ± 20	0.001
Diastolic blood pressure (mm Hg)	82 ± 11	87 ± 9	0.008
BMI (kg/m^2)	26.0 ± 3.8	28.4 ± 4.8	0.001
Plasma glucose (mmol/l)	5.7 ± 0.7	9.0 ± 2.4	<0.001
Insulin (mU/l)	6.8 (4.8-8.7)	9.7 (6.6-15.2)	<0.001
$HOMA_{ir}$ (mU × mmol/l^2 × 22.5)	1.61 (1.14-2.36)	3.95 (2.37-6.12)	<0.001
HbA1c (mmol/mol)	35 ± 3	51 ± 9	<0.001
TSH (mU/l)	1.65 ± 0.61	1.56 ± 0.77	0.42
Free T_4 (pmol/l)	13.6 ± 1.42	14.2 ± 1.57	0.016
TSHI (mU x pmol/l^2)	2.02 ± 0.24	2.05 ± 0.30	0.47
Total cholesterol (mmol/l)	5.74 ± 0.96	5.41 ± 0.94	0.036
Non-HDL cholesterol (mmol/l)	4.25 ± 1.03	4.16 ± 1.03	0.62
LDL cholesterol (mmol/l)	3.53 ± 0.87	3.30 ± 0.84	0.10
HDL cholesterol (mmol/l)	1.49 ± 0.42	1.25 ± 0.37	<0.001
Triglycerides (mmol/l)	1.31 (0.91-1.92)	1.76 (1.17-2.46)	0.029
TNF-α (ng/l)	3.00 (2.35-3.85)	3.50 (2.80-4.90)	0.007

Data are means ± SD and medians (interquartile ranges) and numbers. BMI: body mass index; free T4: free thyroxine; HOMAir: homeostasis model assessment of insulin resistance; HbA1c: glycated hemoglobin; HDL: high density lipoproteins; LDL: low density lipoproteins; non-HDL: non-high density lipoproteins; TSH: thyroid stimulating hormone. TSHI: TSH index. LDL cholesterol was calculated in 79 non-diabetic subjects and in 69 T2DM subjects.

Univariate analyses showed that TNF-α was correlated positively with insulin and HOMAir in non-diabetic subjects (Table II). In T2DM subjects, TNF-α was not significantly related to any of the clinical and metabolic variables listed in Table II. In all subjects combined, there were positive correlations of TNF-α with insulin, glucose, HOMAir and triglycerides, and an inverse correlation with HDL cholesterol.

Table 2. Univariate correlations of plasma tumor necrosis factor alpha (TNF-α) with clinical characteristics and metabolic variables in 81 non-diabetic subjects, in 73 subjects with Type 2 diabetes mellitus (T2DM) and in all subjects combined (n=154).

	Non-diabetic subjects (n=81)	**T2DM subjects** (n=73)	**All subjects combined** (n=154)
	TNF-α	TNF-α	TNF-α
Age	-0.027	0.186	0.105
Systolic blood pressure	0.062	0.120	0.148
Diastolic blood pressure	-0.004	0.056	0.067
BMI	0.122	0.016	0.120
Insulin	0.271**	0.073	0.233***
Glucose	0.076	0.107	0.211***
$HOMA_{ir}$	0.279**	0.102	0.271***
HbA1c	-0.009	0.022	0.151
Total cholesterol	-0.083	0.074	-0.043
Non-HDL cholesterol	-0.023	0.111	0.033
LDL cholesterol	-0.085	0.022	0.061
HDL cholesterol	-0.134	-0.123	-0.182*
Triglycerides	0.131	0.185	0.190**

Pearson correlation coefficients are shown. HOMAir: homeostasis model assessment of insulin resistance; HbA1c: glycated hemoglobin; LDL: low density lipoproteins; non-HDL: non-high density lipoproteins.

*TNF-α, insulin, HOMAir and triglycerides are logarithmically transformed. LDL cholesterol was calculated in 79 non-diabetic subjects and in 69 T2DM subjects. *p < 0.05; **p≤ 0.02; ***p<0.01; ****p≤ 0.001.*

In non-diabetic subjects, TNF-α was correlated inversely with free T_4 (r = -0.254, p = 0.022), but such a relationship was absent in T2DM subjects (r=0.058, p = 0.63; Table III; Figure 1). In non-diabetic subjects, this relationship was also present after exclusion of subjects with thyroid autoantibodies (r = -0.280, p = 0.015). In T2DM subjects, total cholesterol, non-HDL cholesterol and triglycerides were correlated positively with TSH. In all subjects combined, total cholesterol and non-HDL cholesterol were also correlated positively with TSH. TNF-α was not correlated with TSHI in diabetic (r=0.080, p = 0.50) and in non-diabetic subjects (r = -0.088, p = 0.44).

Multivariable linear regression analyses were performed to determine the independent association of TNF-α with free T_4 taking age and sex into account (Table IV). In this analysis, TNF-α was associated inversely with free T_4 in non-diabetic subjects, but not in T2DM subjects (model 1). In non-diabetic subjects, this association was essentially similar after further adjustment for $HOMA_{ir}$ (β = -0.211, p = 0.058). In T2DM subjects, there was still no association of TNF-α with free T_4 after adjustment for $HOMA_{ir}$, and the use of glucose lowering and antihypertensive medication (β = 0.084, p = 0.52), as well as after exclusion of subjects using metformin (β = 0.104, p = 0.59) or antihypertensive medication (β = 0.123, p = 0.47). Furthermore, the association of TNF-α with free T_4 was different in non-diabetic subjects compared to diabetic subjects as indicated by the statistical significance of the interaction term of diabetes status with free T_4 impacting on TNF-α (model 2). This interaction was also present after further adjustment for BMI, blood pressure, $HOMA_{ir}$ and lipids (interaction term: p = 0.027; data not shown).

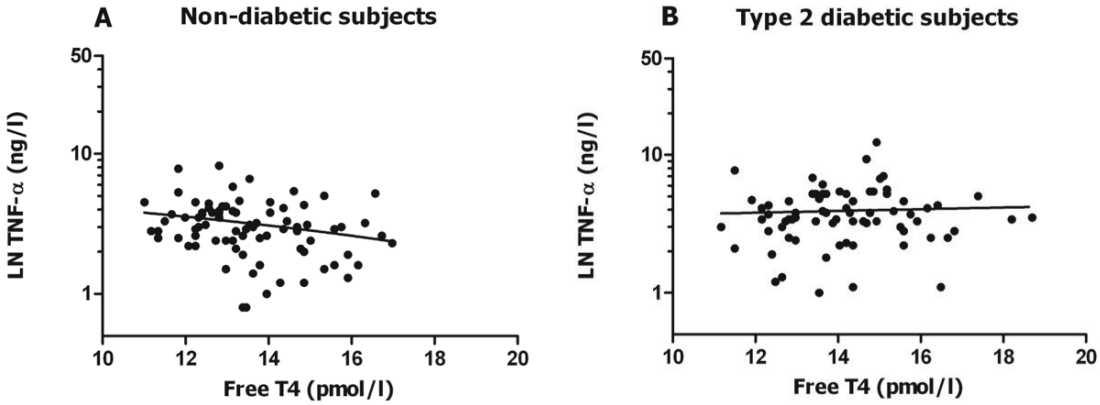
Table 3. Univariate correlations of thyroid function parameters with clinical characteristics, metabolic variables and plasma tumor necrosis factor alpha (TNF-α) in 81 non-diabetic subjects, 73 subjects with Type 2 diabetes mellitus (T2DM) and in all subjects combined (n=154).

	Non-diabetic subjects (n=81)		**T2DM subjects** (n=73)		**All subjects combined** (n=154)	
	TSH	Free T_4	TSH	Free T_4	TSH	Free T_4
Age	0.039	0.087	-0.161	0.170	-0.074	0.155
Systolic blood pressure	-0.099	-0.081	-0.166	0.038	-0.148	0.038
Diastolic blood pressure	-0.147	0.010	0.067	-0.015	-0.052	0.040
BMI	-0.029	-0.126	0.137	-0.300	0.048	-0.156
Insulin	-0.099	-0.156	0.020	-0.203	-0.054	-0.098
Glucose	0.057	-0.022	-0.100	-0.176	-0.092	0.045
HOMAir	-0.084	-0.153	-0.025	-0.233	-0.078	-0.051
Total cholesterol	0.094	0.024	0.282**	0.049	0.200**	0.002
Non-HDL cholesterol	0.034	0.048	0.282**	0.038	0.170*	-0.080
LDL cholesterol	0.060	0.026	0.180	0.173	0.132	0.065
HDL cholesterol	0.133	-0.063	-0.072	0.020	0.043	-0.080
Triglycerides	-0.017	0.081	0.243*	-0.183	0.125	-0.028
TNF-α	0.141	-0.254*	0.018	0.058	0.058	-0.051

Pearson correlation coefficients are shown. Free T4: free thyroxine; HDL: high density lipoproteins; HOMAir: homeostasis model assessment of insulin resistance; LDL: low density lipoproteins; non-HDL: non-high density lipoproteins; TSH: thyroid-stimulating hormone.

*TNF-α, insulin, HOMAir and triglycerides are logarithmically transformed. LDL cholesterol was calculated in 79 non-diabetic subjects and in 69 T2DM subjects. *p < 0.05; **p ≤ 0.02.*

Figure 1.



A. Relationship of tumor necrosis factor-α (TNF-α) with free thyroxine (free T4) in non-diabetic subjects (n=81; r = -0.254, $p = 0.022$).

B. Relationship of TNF-α with free T4 in Type 2 diabetic subjects (n=73, r = 0.058, $p = 0.63$).

Table 4. Multivariable linear regression analyses demonstrating relationships of tumor necrosis factor alpha (TNF-α) with free thyroxine (free T4) in 81 non-diabetic subjects and73 subjects with Type 2 diabetes mellitus (T2DM), and the interaction term of diabetes status with free T4 on TNF-α in all subjects combined (n=154).

	Non-diabetic subjects (n=81)		**Diabetic subjects (n=73)**		**All subjects (n=154)**	
	Model 1		**Model 1**		**Model 2**	
	β	*p-value*	β	*p-value*	β	*p-value*
Age	-0.027	0.81	0.180	0.83	0.077	0.34
Sex (men/women)	0.076	0.51	-0.012	0.14	0.014	0.87
T2DM (yes/no)					-1.217	0.103
Free T_4	-0.243	0.032	0.026	0.83	-0.268	0.023
T2DM x freeT_4					1.486	0.053

β: standardized regression coefficient. TNF-α is logarithmically transformed.
Models 1: *T2DM and non-diabetic subjects separately adjusted for age and sex*
Model 2: *T2DM and non-diabetic subjects combined adjusted for age, sex and diabetes status, and including the interaction term of T2DM with free T4 (T2DM x free T4) to indicate the difference in the relationship of TNF-α with free T4 between T2DM and non-diabetic subjects.*

Discussion

This study has shown-to our knowledge for the first time-that plasma TNF-α is inversely correlated with free T_4 in non-diabetic subjects. This relationship remained present after adjustment for age and sex. In view of the well delineated role of TNF-α as a pro-inflammatory mediator [18], the present data raise the possibility that low-normal thyroid function may contribute to enhanced low-grade chronic inflammation.

Based on findings in SCH it has been proposed that thyroid hormone function may influence pro-inflammatory biomarkers [12,23,24,30]. Accordingly, SCH may relate to higher circulating TNF-α and interleukin-6 levels [24,30]. The presently observed inverse relationship of TNF-α with free T_4 extend these findings in SCH, and are consent with the hypothesis that effects of thyroid function status on pro-inflammatory pathways are likely to extend in the euthyroid range. The regulatory mechanisms whereby low-normal thyroid function relates to higher TNF-α levels are not precisely understood. TSH may stimulate TNF-α secretion from adipocytes [31], whereas an effect of TNF-α on thyroid hormone receptor expression has also been proposed [32,33]. However, we did not observe a relation of either TSH or the TSHI, reflecting the set-point of TSH feedback regulation by thyroid hormone [27,28], with TNF-α. Such regulatory pathways [31-33] probably do not fully explain the inverse correlation of TNF-α with free T_4 in non-diabetic subjects, and the absence of a relationship with TSH as observed in diabetic subjects. Additionally, we cannot exclude that TNF-α would have had a negative impact on T_4 regulation, although the inclusion of apparently healthy non-diabetic individuals makes a contribution of a non-thyroidal illness phenomenon unlikely.
lthy non-diabtic individuals makes a a non-thyroidal illness phenomenon could have contributed to the relationship of
TNF-α has been reported to be involved in the pathogenesis and progression of atherosclerosis, myocardial ischemia/reperfusion injury and heart failure [19,34]. Higher TNF-α levels in the context of low-normal thyroid function could therefore have functional consequences. In line, endothelial dysfunction relates to TNF-α in SCH [30]. Furthermore, inflammatory activity in atherosclerotic lesions from SCH patients is probably exaggerated in conjunction with circulating higher TNF-α levels [24]. Of further relevance, TNF-α relates positively with plasma triglycerides [35,36], as confirmed in the present study. Low-normal thyroid function is featured by pro-atherogenic elevations in large triglyceride-rich lipoprotein [37], which are considered to play a central role in the pathogenesis of low HDL cholesterol [38-41]. Notably, it was recently found that TNF-α may directly influence lipid metabolism via the proprotein convertase subtilisin-kexin type 9 (PCSK9) pathway [42]. This pathway plays a key role in LDL receptor expression [42], whereas circulating PCSK9 correlates not only with LDL cholesterol but also with triglycerides and triglyceride-rich intermediate density lipoproteins [43]. It may, therefore, be proposed that possible effects of higher TNF-α on triglyceride-rich lipoprotein metabolism may contribute to effects of TNF-α on accelerated atherosclerosis.

Plasma TNF-α levels were elevated in T2DM subjects as expected [44]. In the current study, free T_4 was slightly higher in T2DM subjects but TSH level were not different between diabetic and non-diabetic subjects, in line with some previous reports [41,45]. Of note, the TSHI was similar in the groups questioning pathophysiological relevance of the minor free T_4 elevations in T2DM. Remarkably, the relationship of TNF-α with free T_4 was absent in diabetic subjects. The reasons for the absence of such a relationship are unclear at present, but could point to a disturbed thyroid function-TNF-α interrelation in the context of chronic hyperglycemia. Metformin treatment may influence pituitary-thyroid hormone feedback regulation as supported by a lower TSH level despite unaltered thyroid hormone levels [46,47]. However, no independent effect of metformin therapy on the TSH level was found in euthyroid T2DM subjects [47]. In the current study, there was neither a relation of free T_4 with TNF-α in diabetic subjects taking account of the use of metformin, nor after exclusion of metformin using participants. This suggests that the use of metformin did not obscure the lack of a relationship of thyroid functional status with TNF-α in diabetic individuals.

Several methodological issues and limitations of our study need to be described. First, since we performed a cross-sectional study, cause-effect of relationships of TNF-α with thyrroid hormone levels cannot be unequivocally established. Second, we only measured free T_4, thus precluding to assess the dynamics of thyroid hormone metabolism. However, variations in free T_4 in the context of differences in TSH within the euthyroid range are more outspoken than variations in free T_3 [48], making it less likely that free T_3 measurement would have provided important additional information regarding the possible association between variation of thyroid function within the euthyroid range and TNF-α. Third, we excluded statin using subjects in view of decreasing effects of statins on circulating TNF-α [49]. Fourth, a considerable number of diabetic subjects used antihypertensive medication, but this did not obscure the lack of relationship of TNF-α with thyroid function in this patient category. Finally, although thyroid auto-antibodies were not available in diabetic subjects, in non-diabetic subjects a similar inverse correlation of TNF-α with free T_4 was found after exclusion of participants with thyroid auto-antibodies, suggesting that latent thyroid autoimmunity did not play a major role the association of TNF-α lower free T_4.

In conclusion, this study shows that lower free T_4 levels confer higher levels of TNF-α, suggesting that thyroid functional status within the euthyroid range could influence pro-inflammatory pathways. This relationship appears to be disturbed in T2DM. Our findings warrant prospective evaluation with respect to the extent to which subclinical and overt hypothyroidism and its treatment affects pro- and anti-inflammatory cytokines.

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8.

Increased leptin/adiponectin ratio relates to low-normal thyroid function in metabolic syndrome

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Abstract

Background: Low-normal thyroid function within the euthyroid range may contribute to increased atherosclerosis susceptibility. The leptin/adiponectin (L/A) ratio is associated with cardiovascular disease and reflects adipose tissue dysfunction. Relationships of the L/A ratio with low-normal thyroid function are unknown.

Methods: Relationships of thyroid stimulating hormone (TSH) and free thyroxine (free T_4) with leptin, adiponectin and the L/A ratio in euthyroid subjects were documented in 67 fasting subjects with metabolic syndrome (Mets) and 86 euthyroid subjects without MetS (TSH and free T_4 levels within the institutional reference range).

Results: Neither plasma leptin nor adiponectin was significantly correlated with TSH or free T_4 in subjects with and without MetS. In the whole group, high sensitivity C-reactive protein (hs-CRP) was positively correlated with the L/A ratio ($r = 0.485$, $P<0.001$). Notably, the L/A ratio was positively correlated with TSH in subjects with MetS ($r=0.252$, $P=0.040$) but not in subjects without MetS ($r=-0.068$, $P=0.54$; interaction term, $P=0.027$). In MetS subjects, the L/A ratio remained positively related with TSH after adjustment for age, sex, diabetes status, hs-CRP and the use of antihypertensive and glucose lowering medication ($\beta = 0.283$, $P=0.018$), as well as after adjustment for individual MetS components ($\beta = 0.294$, $P=0.020$).

Conclusions: In the context of MetS, a higher TSH within the euthyroid range confers an increased L/A ratio, a proposed marker of atherosclerosis susceptibility and adipocyte dysfunction.

Background

The concept is emerging that low-normal thyroid function, as inferred from a higher thyroid stimulating hormone (TSH) or a lower free thyroxine (free T_4) within the euthyroid range, may adversely impact several health issues including the development of cardiovascular disorders [1,2]. In line with this concept, low-normal thyroid function is associated with a greater increased intima media thickness (cIMT), an established biomarker of subclinical atherosclerosis [3,4]. Furthermore, low-normal thyroid function is associated with increased coronary artery calcification [5] and progression thereof [6], although an association of a high-normal TSH level with increased coronary heart disease risk has been variably reported [7,8].

Several factors are likely to contribute to the association of (subclinical) atherosclerosis with low-normal function. Low-normal thyroid function relates to higher plasma levels of total cholesterol and atherogenic apolipoprotein B-containing lipoproteins [9], and contributes to enhanced cholesteryl ester transfer from HDL to triglyceride-rich lipoproteins, a pro-atherogenic process [10,11]. Low-normal thyroid function conveys changes in high density lipoprotein (HDL) anti-oxidative function as well, which conceivably contribute to impaired oxidative stress defense [12]. Interestingly, thyroid function status affects circulating levels of leptin and adiponectin, adipokines which exert pro- and anti-atherogenic properties, respectively [13,14,15]. Thus, leptin has been reported to decrease and adiponectin to increase after levothyroxine supplementation in subclinical hypothyroidism [16]. These findings provide a rationale to hypothesize that the plasma leptin/adiponectin (L/A) ratio is higher in subjects with low-normal thyroid function. Of note, the L/A ratio may represent a preferential marker compared to leptin and adiponectin alone in predicting incident cardiovascular disease [17,18]. The L/A ratio is also considered to represent a biomarker of adipocyte dysfunction [19]. Higher plasma leptin and lower adiponectin levels are well known features of the metabolic syndrome (MetS) [20]. As a result, the L/A ratio is elevated in MetS [21,22,23], which supports the potential clinical relevance to determine relationships of low-normal thyroid function with the L/A ratio in subjects with MetS.

We initiated the present study to determine possible relationships of plasma leptin, adiponectin and the L/A ratio with TSH and free T_4 in euthyroid subjects with and without MetS.

Methods

Subjects

The study protocol was approved by the medical ethics committee of the University Medical Center Groningen, the Netherlands, approved the study. Written informed consent was obtained from the participants, who were aged > 18 years. Type 2 Diabetes Mellitus (T2DM) and non-diabetic subjects were approached by advertisement in local newspapers. T2DM had been diagnosed previously by primary care physicians applying a fasting plasma glucose ≥ 7.0 mmol/l and/or non-fasting plasma glucose ≥11.1 mmol/l as diagnostic criteria. MetS was defined according to NCEP-ATP III criteria [24]. Three or more of the following criteria were required for categorization of subjects with MetS: waist circumference >102 cm for men and >88 cm for women; hypertension (blood pressure ≥ 130/85 mm Hg or use of antihypertensive drugs); fasting plasma triglycerides ≥ 1.7 mmol/l; HDL cholesterol < 1.0 mmol/l for men and <1.3 mmol/l for women; fasting glucose ≥ 5.6 mmol/l.

Serum TSH and free T_4 levels had to be within the institutional reference range, and anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-Tg) auto-antibodies had to be absent (see below).

Subjects with a history of cardiovascular disease (CVD), chronic kidney disease (estimated glomerular filtration rate < 60 ml/min/1.73 m^2 or micro/macroalbuminuria), liver disorders (serum transaminase levels >2 times the upper reference limit), as well as current smokers, subjects who used lipid lowering drugs or insulin were also excluded from participation as were pregnant women. The use of anti-hypertensive medication and oral contraceptives was allowed.

Physical examination did not reveal pulmonary or cardiac abnormalities. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Blood pressure was measured after 15 min of rest at the left arm using a sphygmomanometer. The participants were evaluated between 0800 and 1000 h after an overnight fast.

Laboratory analyses

Serum and EDTA-anticoagulated plasma samples, prepared by centrifugation at 1400 g for 15 min at 4^0C, were stored at -80 ^{0}C until analysis. Plasma glucose was measured shortly after blood collection.

Serum TSH (sandwich principle; Roche Diagnostics GmbH., Mannheim, Germany, cat. no. 117314591; reference range 0.5-4.0 mU/l) and free T_4 (competition principle; Roche Diagnostics GmbH., Mannheim Germany, cat. no. 11731297; reference range 11.0-19.5 pmol/l) were measured with a electrochemiluminescence immunoassay on a Modular Analytics immunoassay analyzer. Anti-TPO and anti-Tg) auto-antibodies were measured with enzyme-linked immunoassays (ImmunoCap cat nos. 14-4508-35 and

14-4507-35, respectively; Phadia, Freiburg, Germany), and were considered to be positive using cut-off values provided by the supplier (anti-TPO antibodies > 60 IU/ml and anti-Tg antibodies > 289 IU/ml).

Plasma leptin and total adiponectin were assayed using commercially available assays (Luminex xMAP technology; Linco Research Inc., St Charles, MO, USA; Lincoplex panel A cat. no. HADK1-61K-A and panel B cat. no. HADK2-61K-B) [25]. All intra-assay and inter-assay coefficients of variation were <6 % and <8 %. High sensitivity C-reactive protein (hs-CRP) was determined was by nephelometry (BNII N; Dade Behring, Marburg Germany) [17].

Plasma total cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos 11875540 and 11876023, respectively; Roche Diagnostics GmbH, Mannheim, Germany). HDL cholesterol was measured with a homogeneous enzymatic colorimetric test (Roche/Hitachi, cat no 04713214; Roche Diagnostics GmbH, Mannheim, Germany). Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. Glucose was measured on an APEC glucose analyzer (APEC Inc., Danvers, MA, USA).

Statistical analysis

SPSS 22 (version 22.0, SPSS Inc., Chicago, IL, USA) was used for data analysis. Data are expressed as mean ± SD, median (interquartile ranges) or in numbers. Differences between people with and without MetS were determined by unpaired T-tests or Chi-square tests. Because of skewed distribution, triglycerides, hs-CRP, leptin, adiponectin and the L/A ratio were logarithmically transformed to compare between-group differences, and to perform correlation analyses.

Univariate relationships were calculated using Pearson correlation coefficients. Multivariable linear regression analyses were carried out to disclose the independent relationships of the L/A ratio with thyroid function parameters. To determine whether the relationships of thyroid function parameters with the L/A ratio were different between subjects with and without MetS, interaction terms were calculated as the product term between the thyroid function variable of interest and the presence of MetS. Two-sided *P*-values < 0.05 indicated statistical significance.

Results

We included 67 subjects with MetS and 86 subjects without MetS (Table 1). Forty nine subjects with MetS and 24 subjects without MetS were diagnosed with T2DM (*P*<0.001). Twenty three subjects with MetS and 7 subjects without MetS were taken anti-hypertensive drugs (mostly angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists and diuretics, alone or in combination) (*P*<0.001). Metformin and sulfonylurea were used, either alone or in combination, by 28 and 27 subjects with MetS, respectively. Of the subjects without MetS, 10 used metformin and 12 used sulfonylurea. Other glucose lowering drugs were not used. Estrogens were taken by 2 post-menopausal woman with MetS and by 1 pre-menopausal woman without MetS.

Table 1. Clinical characteristics, glucose, lipids, leptin, adiponectin, the leptin/adionectin (L/A) ratio and thyroid function parameters in 67 subjects with metabolic syndrome (MetS) and in 86 subjects without MetS.

	MetS (n=67)	**No MetS** (n=86)	*P*-value
Age (years)	58 ± 9	56 ± 9	0.10
Sex (men/women)	40/27	52/34	0.92
Systolic blood pressure (mm Hg)	144 ± 18	131 ± 20	<0.001
Diastolic blood pressure (mm Hg)	89 ± 9	81 ± 10	<0.001
BMI (kg/m^2)	29.8 ± 4.4	25.1 ± 3.3	<0.001
Waist (cm)	105 ± 12	87 ± 11	<0.001
Glucose (mmol/l)	8.6 ± 2.7	6.2 ± 1.4	<0.001
Total cholesterol (mmol/l)	5.54 ± 0.97	5.62 ± 0.97	0.60
Non-HDL cholestrerol (mmol/l)	4.38 ± 0.98	4.07 ± 1.05	0.068
HDL cholesterol (mmol/l)	1.16 ± 0.34	1.55 ± 0.38	<0.001
Triglycerides (mmol/l)	1.96 (1.70-2.52)	1.15 (0.85-1.55)	<0.001
hs-CRP (mg/l)	2.03 (1.26-4.22)	1.02 (0.48-2.25)	<0.001
Leptin (µg/l)	11.5 (5.5-31.9)	4.8 (3.0-11.6)	<0.001
Adiponectin (mg/l)	14.7 (9.2-26.8)	18.2 (14.2-38.8)	<0.001
L/A ratio (µg/mg)	0.83 (0.37-1.85)	0.23 (0.12-0.62)	<0.001
TSH (mU/l)	1.55 ± 0.70	1.66 ± 0.68	0.37
Free T_4 (pmol/l)	13.9 ± 1.59	13.8 ± 1.47	0.82

Data in mean ± SD or in median (interquartile range). BMI, body mass index; hs-CRP, high sensitivity C-reactive protein; TSH, thyroid stimulating hormone; free T4, free thyroxine. Logarithmically transformed values of triglycerides, hs-CRP, leptin, adiponectin and the L/A ratio are used for statistical comparisons.

Age, sex distribution, TSH and free T_4 levels were not significantly different between subjects with and without MetS (Table 1). Blood pressure, BMI, waist, plasma glucose and triglycerides were expectedly higher, whereas HDL cholesterol was lower in MetS subjects. Total cholesterol and non-HDL cholesterol were not significantly different between the groups. Plasma hs-CRP was elevated in MetS subjects. Furthermore, leptin was increased and adopinectin was decreased in MetS subjects (Table 1). As a result, the L/A ratio was approximately three-fold higher in MetS subjects.

In the whole group, hs-CRP was correlated positively with leptin (r=0.435, *P*<0.001) and inversely with adiponectin in univariate analysis (r=-0.289, *P*<0.001). Consequently, hsCRP was correlated positively with the L/A ratio (r=0.485, *P*<0.001). In the whole group, neither plasma leptin nor adiponectin or the L/A ratio were significantly correlated with TSH or with and free T_4 (*P*>0.40 for all; data not shown). Furthermore, neither plasma leptin nor adiponectin were significantly correlated with TSH and free T_4 in subjects with or without MetS (Table 2). However, the L/A ratio was positively correlated with TSH in MetS subjects, contrasting the lack of such a relationship in subjects without MetS (Table 3; Figure 1). Indeed, the relationship of the L/A ratio with TSH was different between subjects with and without MetS (interaction term for between group difference: β=0.201, *P*=0.027). The positive relation of the L/A ratio with TSH was also present in MetS subjects who did not use antihypertensive drugs (n=43; r=0.329, *P*=0.034) or metformin (n=39; r=0.285, *P*=0.070), again without such relationships being found in subjects without MetS (n=79; r=-0.026, *P*=0.82 and n=76, r=-0.047, *P*=0.69, respectively).

Table 2. Univariate relationships of leptin, adiponectin and the leptin/adiponectin (L/A) ratio with thyroid stimulating hormone (TSH) and free thyroxine (free T4) in 67 subjects with metabolic syndrome (MetS) and in 86 subjects without MetS.

	MetS (n=67)		**No MetS** (n=86)	
	TSH	free T_4	TSH	free T_4
Leptin	0.168	0.020	-0.013	-0.113
Adiponectin	-0.090	-0.046	0.154	0.056
L/A ratio	0.252*	0.031	-0.068	-0.106

*Pearson correlation coefficients are shown. Logarithmically transformed values of leptin, adiponectin, the L/A ratio are used for statistical comparisons. *P=0.04.*

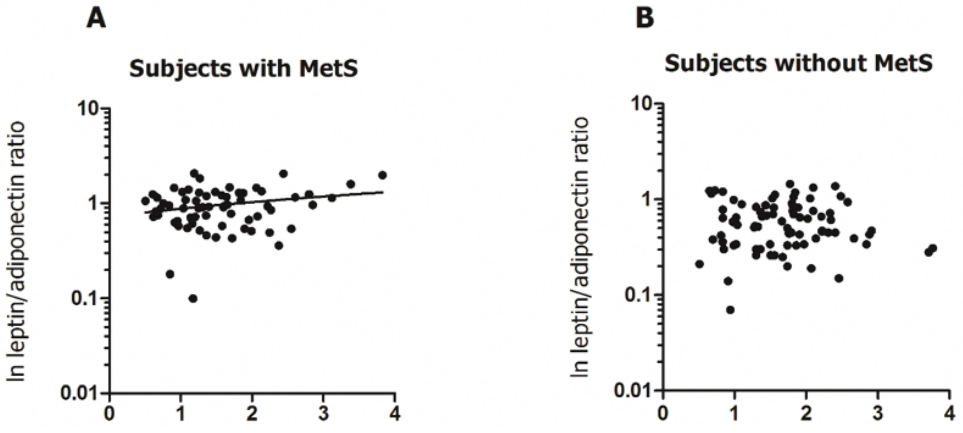
Table 3. Multivariable linear regression analysis demonstrating the relationship of the leptin/adiponectin (L/A) ratio with thyroid stimulating hormone (TSH), age, sex and diabetes status and high sensitivity C-reactive protein (hs-CRP) in 67 subjects with metabolic syndrome (MetS; A) and in 86 subjects without MetS (B).

A MetS (n=67)	L/A ratio	
	β	*P*-value
Age	-0.130	0.25
Sex (women vs. men)	0.278	0.018
T2DM	-0.049	0.74
hs-CRP	0.150	0.21
TSH	0.289	0.018

B No MetS (n=86)	L/A ratio	*P*-value
	β	
Age	-0.076	0.39
Sex (women vs. men)	0.431	<0.001
T2DM	0.002	0.98
hs-CRP	0.430	<0.001
TSH	-0.119	0.22

T2DM: type 2 diabetes mellitus; β: standardized regression coefficient. The L/A ratio and hs-CRP are logarithmically transformed. Models are additionally adjusted for the use of antihypertensive medication, sulfonylurea and metformin.

Figure 1.



A. Relationship of the leptin/adiponectin (L/A) ratio with thyroid stimulating hormone (TSH) in 67 subjects with metabolic syndrome (MetS) (r = 0.252, *P* = 0.04).

B. Relationship of the L/A ratio with TSH in 86 subjects without (MetS (r = -0.068, *P* = 0.54).

Multivariable linear regression analysis demonstrated that the positive relationship of the L/A ratio with TSH in MetS subjects remained present after adjustment for age, sex, diabetes status and the use of antihypertensive and glucose lowering medication (Table 3A). No such relationship was found in subjects without MetS (Table 3B). In alternative analysis, the L/A ratio was also positively related to TSH in MetS subjects when taking the individual MetS components into account and thus being associated with TSH independent of an enlarged waist (Table 4A). Additionally, the L/A ratio was positively related to an enlarged waist in subjects without MetS (Table 4A and 4B). In the whole group, the L/A ratio was independently associated with female sex (β=0.338, *P*<0.001), hs-CRP (β=0.262, *P*<0.001) and an enlarged with waist (β=0.204, *P*=0.005) without an independent association with TSH (β=0.079, *P*=0.214; data not shown).

Table 4. Multivariable linear regression analysis demonstrating the relationship of the leptin/adiponectin (L/A) ratio with thyroid stimulating hormone (TSH), age, sex, metabolic syndrome (MetS) components and high sensitivity C-reactive protein (hs-CRP) in 67 subjects with MetS (A) and in 86 subjects without MetS (B).

A MetS (n=67)	L/A ratio	*P*-value
	β	
Age	-0.154	0.19
Sex (women vs. men)	0.214	0.082
Elevated glucose	0.053	0.69
Hypertension	0.138	0.25
Enlarged waist	0.201	0.12
Elevated triglycerides	-0.064	0.61
Low HDL cholesterol	0.172	0.17
hs-CRP	0.090	0.47
TSH	0.294	0.020

B No MetS (n=86)	L/A ratio	
	β	*P*-value
Age	-0.028	0.76
Sex (women vs. men)	0.53	<0.001
Elevated glucose	0.097	0.31
Hypertension	0.090	0.38
Enlarged waist	0.310	<0.001
Elevated triglycerides	0.177	0.047
Low HDL cholesterol	0.072	0.38
hs-CRP	0.299	<0.001
TSH	-0.060	0.48

HDL: high density lipoproteins; β: standardized regression coefficient. The L/A ratio and hs-CRP are logarithmically transformed. Models are additionally adjusted for the use sulfonylurea and metformin.

Discussion

This study reveals to our knowledge for the first time that the plasma L/A ratio is positively related to a higher TSH level in euthyroid subjects with MetS but not in subjects without MetS. This relationship in MetS subjects remained present when taking relevant covariates into account, including the presence of diabetes, hs-CRP and the use of antihypertensive and oral glucose lowering drugs, and was also present in analysis in which we adjusted for individual MetS components, including an enlarged waist circumference. The current results, therefore, suggest that the L/A ratio, an alleged predictor of cardiovascular disease and biomarker of adipocyte dysfunction [17,19] associates with low-normal thyroid function. Altogether, the present findings add to accumulating evidence which underscores the possibility that low-normal thyroid function may confer increased atherosclerosis susceptibility [1,2,9].

We enrolled strictly euthyroid subjects, as inferred from TSH and free T_4 levels within the institutional reference range. With this selection criterion, TSH and free T_4 were similar in subjects with MetS compared to subjects without MetS. This entirely agrees with our previous findings in a small group of non-diabetic subjects [26], although mild thyroid function changes in MetS have been documented in another study [9]. Leptin and adiponectin play an important role in obesity-associated metabolic risk by modulating inflammatory processes and affecting insulin sensitivity [19,20,27]. In agreement, we found that the L/A ratio was positively related to hs-CRP in univariate analysis. Given the associations of plasma leptin and adiponectin with (central) obesity, the strong elevations in the L/A ratio in MetS subjects as demonstrated here is not surprising [21,22,23]. Accordingly, waist circumference predicted the L/A ratio in the present study, even independent of hyperglycemia and other MetS components. We consider this finding reassuring because a considerable number of participants had been diagnosed with type 2 diabetes, making that the number subjects without diabetes was too low to allow for meaningful subgroup analysis.

Clinical observations showing that plasma leptin decreases whereas adiponectin increases after levothyroxine substitution in subjects with subclinical hypothyroidism [16] prompted us to delineate the relationship of the L/A ratio with low-normal thyroid function. In concert with these human findings [16], thyroid hormone upregulates adiponectin gene expression in rat adipose tissue [28]. In contrast, leptin gene expression in rat epididymal fat is downregulated after experimental hyperthyroidism, although lower circulating leptin levels in response to high thyroid hormone exposure are at least in part attributable to a decrease in body fat [29]. It remains to be more precisely determined why there was only a relation of the L/A ratio with TSH in the MetS subjects. In comparison,

the relationship with low-normal thyroid function with other pro-atherogenic biomarkers have been demonstrated previously to be particularly evident in diabetic or MetS subjects [2,9,10]. Our present observation that this relationship remained present after adjustment for waist circumference would be consistent with a contribution of thyroid function status on this ratio.

A number of other limitations and methodological aspects of our study need to be discussed. First, we performed a cross-sectional study, making that cause-effect relationships cannot be established with certainty. However, we are not aware of published data indicating that the leptin or adiponectin directly affect thyroid hormone regulation. Second, we relied on a single set of thyroid function parameters. In this regard it is noteworthy that each individual probably has a narrow set-point of thyroid function status, underscoring the pathophysiological relevance of once measured thyroid function status [30]. Third, circulating adiponectin increases in response to angiotensin II, making that an effect of the antihypertensive medication used cannot by excluded [31]. Fourth, metformin lowers of the TSH level in hypothyroid subjects and thus could alter the set-point of the pituitary-thyroid axis [32,33]. Notably, metformin treatment does not elicit TSH changes in euthyroid subjects [33,34]. For these reasons, we adjusted for the use of antihypertensive and glucose lowering drugs in multivariable regression analysis, confirming the independent relation of the L/A ratio with TSH in MetS. In addition, the positive relation of the L/A ratio with TSH in MetS was also present after exclusion of subjects taking antihypertensive medication or metformin, indicating that the use of these medications did not confound the interpretation of our data.

The interest for a neutraceutical approach to improve the cardiometabolic risk profile besides well-established pharmacological treatment modalities is growing [35]. In this respect it is noteworthy that a combination of several compounds including red yeast rice extract, berberine, policosanol, astaxanthin, coenzyme Q10 and folic acid has been shown to reduce the L/A ratio besides low density lipoprotein (LDL) cholesterol lowering [36]. Given the elevated L/A ratio in MetS, additional studies with respect to a neutraceutical approach to ameliorate pro-atherogenic biomarkers appear to be warranted.

Conclusions

A higher TSH level within the euthyroid range confers an increased L/A ratio in MetS subjects, which is likely to contribute to an adverse cardiometabolic profile in this patient category.

List of abbreviations

Anti-thyroid peroxidase: anti-TPO
Anti-thyroglobulin: anti-Tg
Body mass index: BMI
Cardiovascular disease: CVD
High sensitivity C-reactive protein: hs-CRP
Free thyroxine: free T_4
High density lipoprotein: HDL
Intima media thickness: cIMT
Leptin/adiponectin: L/A ratio
Low density lipoprotein: LDL
Metabolic syndrome: MetS
Thyroid stimulating hormone: TSH
Type 2 Diabetes Mellitus: T2DM

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Availability of data and material

Proprietary algorithms used to generate the data in this manuscript will not be made available for general use. Data may be made available by request to the corresponding author.

Author's contributions

R.P.F. Dullaart performed the statistical analyses. R.P.F. Dullaart and L.J.N. van Tienhoven-wind interpreted the data. L.J.N. van Tienhoven-Wind wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

This study is investigator initiated. There are no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The study protocol was approved by the medical ethics committee of the University Medical Center Groningen.

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9.

Higher free triiodothyronine is associated with non-alcoholic fatty liver disease in euthyroid subjects: The Lifelines Cohort Study

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Abstract

Objective: Overt hypothyroidism confers an increased risk of non-alcoholic fatty liver disease (NAFLD). The liver plays a crucial role in the metabolism of cholesterol and triglycerides; thyroid hormones interact on hepatic lipid homeostasis. Thyroid function within the euthyroid range affects a number of health issues, including atherosclerosis development and biochemical markers of increased cardiovascular risk. However, the association of thyroid hormones with NAFLD in euthyroid subjects has not been unequivocally established. We therefore determined associations of thyroid hormone parameters with NAFLD among euthyroid subjects.

Methods: The study was conducted in the Lifelines Cohort Study, a population-based cohort study of participants living in the North of the Netherlands. Only euthyroid subjects (thyroid-stimulating hormone (TSH) 0.5-4.0 mU/L, free thyroxine (FT4) 11-19.5 pmol/L and free triiodothyronine (FT3) 4.4-6.7 pmol/L) older than 18 years were included. Exclusion criteria were participants with excessive alcohol use, known hepatitis or cirrhosis, liver functions ≥ three times the upper limit, current cancer, non-white ancestry, previous or current use of thyroid medication and current use of lipid or glucose lowering medication. A priori defined liver biochemistry, thyroid function parameters and metabolic syndrome (MetS) were studied. NAFLD was defined by using the validated Fatty Liver Index (FLI); FLI ≥ 60 was categorized as NAFLD. A $P<0.01$ was considered significant.

Results: FLI ≥ 60 was found in 4,274 (21.1%) of 20,289 individuals (62.1% male, median age 46 years) with increased prevalence of MetS ($P<0.0001$). In age- and sex-adjusted analysis FLI ≥ 60 was independently associated with a higher FT3 (OR 1.34, 95% CI 1.29-1.39, per SD increment, $P<0.0001$) and a lower FT4 (OR 0.73, 95% CI 0.70-0.75, $P<0.0001$) but not by TSH. The strongest association was found for the FT3/FT4 ratio (OR 1.44, 95% CI 1.39-1.49, $P<0.0001$). These associations remained similar after additional adjustment for the presence of MetS. In subjects with enlarged waist circumference, TSH and FT4 were lower while FT3 was higher, resulting in an increased FT3/FT4 ratio ($P<0.0001$).

Conclusions: Euthyroid subjects with suspected NAFLD are characterized by higher FT3, lower FT4 and higher FT3/FT4 ratio, probably consequent to central obesity.

Key words: euthyroidism**,** free triiodothyronine, free thyroxine, non-alcoholic fatty liver disease, central obesity.

Abbreviations

ALP, alkaline phosphatase; **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **BMI**, body mass index; **CI**, confidence interval; **DIO**, iodothyronine deiodinase; **FLI**, Fatty Liver Index; **FT3**, free triiodothyronine; **FT4**, free thyroxine; **GGT**, gamma-glutamyl transferase; **HDL**, high-density lipoprotein; **IQR**, interquartile range; **MetS**, metabolic syndrome; **NAFLD**, non-alcoholic fatty liver disease; **NASH**, non-alcoholic steatohepatitis; **NCEP ATP III**, National Cholesterol Education Program Adult Treatment Panel III; **NFS**, NAFLD fibrosis score; **OR**, odds ratio; **SD**, standard deviation; **TG**, triglycerides; **TSH**, thyroid-stimulating hormone; **VLDL**, very low density lipoproteins.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as the presence of hepatic steatosis in the absence of excessive alcohol consumption [1]. NAFLD includes a broad spectrum of pathology ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis, while it also predisposes to hepatocellular carcinoma. NAFLD is considered to reflect the hepatic component of the metabolic syndrome (MetS), since there is a strong association with insulin resistance, (central) obesity, dyslipidemia and hypertension [2]. As a consequence of the obesity epidemic, NAFLD is the leading cause of chronic liver disease in the Western world. It is estimated that NAFLD occurs in 20-30% of European adults [3]. Therefore, it is expected that subjects with NAFLD will require further identification of concurrent NASH and/or fibrosis to be a prominent target for lifestyle modification and pharmacological intervention in the near future [4].

The liver plays a crucial role in the metabolism of cholesterol and triglycerides [5], with hepatic fat accumulation being regarded as the driving force of elevated plasma triglycerides [6]. Importantly, thyroid hormones interact on hepatic lipid homeostasis through multiple pathways, including stimulation of free fatty acid delivery to the liver for re-esterification to triglycerides, and increasing fatty acid β-oxidation, thereby affecting hepatic fat accumulation [5,7-10].

Several studies have demonstrated an association between overt thyroid dysfunction and NAFLD. Subjects with hypothyroidism are about 1.5 to 2 times more likely to have biopsy-proven or ultrasonography-confirmed NAFLD [11,12]. Accordingly, a recent longitudinal study demonstrated that (subclinical) hypothyroidism is associated with NAFLD risk [13]. A systematic review indeed suggested a relation between NAFLD and hypothyroidism, although such an effect has not consistently been reported [14]. There are, however, only a few small studies, which aimed to assess the association of NAFLD with variations in thyroid function within the euthyroid range [15-19]. Higher thyroid-stimulating hormone (TSH) levels within the euthyroid range were found in subjects with NAFLD but may also relate to attenuated serum alanine aminotransferase (ALT) elevations in the context of MetS and insulin resistance [15,16]. In euthyroid subjects with NAFLD, lower free thyroxine (FT4) levels were found in some studies [15,17,18], whereas a higher free triiodothyronine (FT3) level was found in middle-aged Chinese subjects [19].

Given the importance of variations in thyroid function within the euthyroid range for a considerable number of health issues, including (subclinical) atherosclerosis and altered levels of pro-atherogenic biochemical markers [5,20], it is relevant to examine the relationship of NAFLD with thyroid function parameters in an euthyroid population. In the present cross-sectional study, we aimed to determine the relationship of NAFLD with TSH, FT4 and FT3 among participants of the Lifelines Cohort Study, representative of the general population from the North-Eastern region of the Netherlands.

Material and methods

2.1 Study design

The present cross-sectional study is conducted in the framework of the Lifelines Cohort Study. The Lifelines Cohort Study is a multi-disciplinary prospective population-based cohort study examining in a unique three-generation design the health and health-related behaviors of 167,729 persons living in the North-Eastern region of the Netherlands. It employs a broad range of investigative procedures in assessing the biomedical, socio-demographic, behavioral, physical and psychological factors which contribute to the health and disease of the general population, with a special focus on multi-morbidity and complex genetics [21]. All participants provided written informed consent. The medical ethics committee of the University of Groningen, the Netherlands, approved the study conforming to the Declaration of Helsinki.

2.2 Participants

We included subjects of Western-European origin and all participants were aged between 18 and 85 years at time of enrollment. Only euthyroid subjects participated in the present study. Euthyroidism was defined as a TSH level between 0.5-4.0 mU/L, FT4 level between 11-19.5 pmol/L and FT3 level between 4.4-6.7 pmol/L, i.e. within their respective institutional reference ranges. Eligible subjects had liver enzyme values < 3 times the upper reference limit, i.e. for aspartate aminotransferase (AST) < 120 U/L, alanine aminotransferase (ALT) < 135 U/L, gamma-glutamyl transferase (GGT) < 165 U/L and alkaline phosphatase (ALP) < 360 U/L. Additional exclusion criteria were: missing data required to calculate the Fatty Liver Index (FLI, as outlined below) and to determine the presence of metabolic syndrome (MetS) and its components and non-white ancestry (participants were assumed to be immigrant if his/her birth country or that of one or both parents was outside the Netherlands). The representativeness of the Lifelines cohort for the North Netherlands population has been previously validated [22]. Further exclusion criteria were: excessive alcohol use (>1 alcoholic drink per day for women and > 2 alcoholic drinks per day for men [23]), previously diagnosed hepatitis and/or cirrhosis, current cancer, previous or current use of thyroid medication and the use of lipid lowering or glucose lowering medication (including oral glucose lowering medication and insulin). Information about nationality, alcohol consumption, hepatitis B virus infection, liver cirrhosis, current cancer and medication use was extracted from the self-administered questionnaires.

2.3 Data collection

Data collection of the Lifelines Cohort Study started in 2006 and is ongoing. Questionnaires were collected, anthropometry and blood pressure were measured and biomaterial (blood) was collected at the Lifelines research sites. A standardized protocol was used

to obtain blood pressure and anthropometric measurements (height, weight and waist circumference). Systolic and diastolic blood pressures were measured 10 times during a period of 10 minutes, using an automated Dinamap Monitor (GE Healthcare, Freiburg, Germany). The size of the cuff was chosen according to the arm circumference. The average of the final three readings was used for each blood pressure parameter. Anthropometric measurements were measured without shoes. Body weight was measured to the nearest 0.5 kg. Height and waist circumference were measured to the nearest 0.5 cm. Height was measured with a stadiometer placing their heels against the rod and the head in Frankfort Plane position. Waist circumference was measured in standing position with a tape measure all around the body at the level midway between the lower rib margin and the iliac crest [21]. Venous blood samples were collected in the fasting state between 8.00 and 10.00 a.m. and collected into heparin-containing tubes, centrifuged at 1,885x*g* and the plasma aliquots were processed for laboratory measurements at the same day and stored at -80 ^{0}C. TSH, FT4 and FT3 were measured by electrochemiluminescent immunoassays on a Roche Modular E170 analyzer, using kits provided by the manufacturer (Roche, Mannheim, Germany). High-density lipoprotein (HDL) cholesterol and triglycerides (TG) were measured using routine procedures on a Roche Modular P chemistry analyzer. Glucose was assayed by the hexokinase method. GGT, ALP, ALT and AST were routinely measured according to the recommendations of the International Federation of Clinical Chemistry on a Roche Modular platform. ALT and AST were measured with pyridoxal phosphate activation. All laboratory measurements were performed at the Department of Laboratory Medicine of the University Medical Center Groningen, the Netherlands [21].

2.4 Definitions and calculations

In order to categorize subjects with a high probability for the diagnosis of NAFLD the Fatty Liver Index (FLI) was used. FLI was calculated according to the formula published by Bedogni [24]. $FLI = (e^{0.953*\log e\ (triglycerides) + 0.139*BMI + 0.718*\log e\ (GGT) + 0.053*waist\ circumference - 15.745}) / (1+e^{0.953*\log e\ (triglycerides) + 0.139*BMI + 0.718*\log e\ (GGT) + 0.053*waist\ circumference - 15.745}) *100$. The optimal cut-off value for the FLI has been documented to be 60 with an accuracy of 0.84, a sensitivity of 61% and a specificity of 86% for detecting NAFLD as determined by ultrasonography [24]. A FLI ≥ 60 was categorized as NAFLD. The 2016 EASL-EASD-EASO NAFLD guideline recommends that for larger scale screening studies, serum biomarkers are the preferred diagnostic with the FLI currently being considered as one of the best-validated steatosis scores [23]. To identify NAFLD patients with advanced fibrosis, the NAFLD fibrosis score (NFS) was used. To calculate the NAFLD fibrosis score the formula published by Angulo *et al.* was used [25]. *NAFLD Fibrosis Score (NFS) = (-1.675 + 0.037 x age (year) + 0.094 x BMI (kg/m^2) + 1.13 x fasting glucose/presence of diabetes (yes = 1, no = 0) + 0.99 x AST/ ALT ratio - 0.013 x platelet count (x10^9/L) – 6.6 x albumin (g/L).* By applying a cutoff score > 0.676, the presence of advanced fibrosis could be diagnosed with a sensitivity of 43%, specificity

of 96% and positive predictive value of 82% [25]. The NFS is currently considered to be one of the best validated biomarkers to diagnose fibrosis among NAFLD subjects [23,26,27].

Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). MetS was defined by the revised diagnostic criteria from the American Heart Association by the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) [28]. The NCEP ATP III criteria consist of five criteria for the MetS: *(1)* an enlarged waist circumference (≥ 102 cm for males and ≥ 88 cm for females); *(2)* elevated triglycerides (TG) (≥ 1.7 mmol/L); *(3)* low HDL cholesterol (< 1.0 mmol/L in males and < 1.3 mmol/L in females); *(4)* elevated blood pressure (systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg) and/or medication use for hypertension; *(5)* elevated fasting plasma glucose (≥ 5.6 mmol/L). Participants were diagnosed with the MetS when at least three out of five criteria were present. Diabetes was defined as a fasting plasma glucose ≥ 7.0 mmol/L.

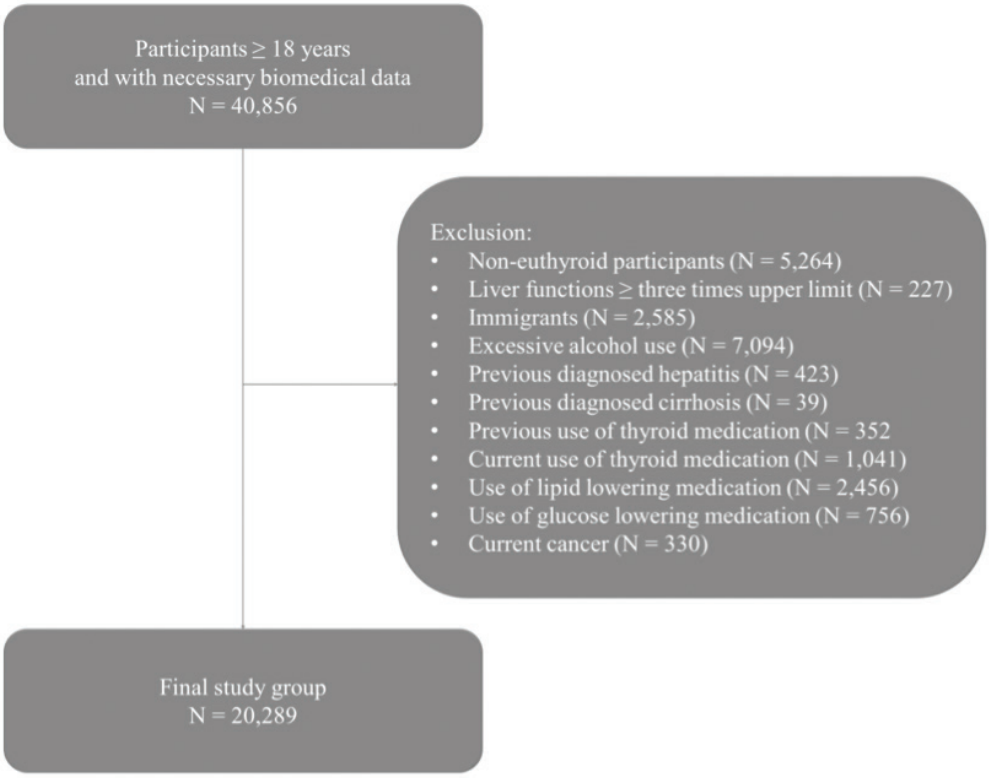
2.5 Statistical Analysis

SPSS 22 (version 22.0, SPSS Inc., Chicago, IL, USA) was used for data analysis. Normality of distribution was assessed and checked for skewness. Descriptive data are expressed in medians with interquartile ranges (IQR) for continuous variables or in numbers with percentages. Between group differences in variables were tested with Mann-Whitney U test and categorical variables were analyzed with Chi-square test. Multivariable logistic regression analysis was performed to disclose the independent association of FLI with thyroid function parameters, MetS and individual MetS components. Separate models were made to determine associations of FT4 and FT3 and the FT3/FT4 ratio with FLI ≥ 60. All models were adjusted for age and sex. For continues variables a Z-score transformation was performed and these standardized variables were used in multivariable analysis. Odds ratios (OR) are given per 1 standard deviation (SD) change in TSH, FT4, FT3 and the FT3/FT4 ratio with 95% confidence intervals (CI). A FLI ≥ 60 and NFS > 0.676 were taken as the optimal cut-off for NAFLD [24] and fibrosis categorization, respectively [25]. In addition, a sensitivity analyses was performed with a FLI ≥ 90 as cut-off, corresponding to a specificity of NAFLD of 99 % [24]. Given multiple comparisons, two-sided *P*-values < 0.01 were considered statistically significant.

Results

Of the whole Lifelines Cohort Study, 40,856 participants were initially considered eligible with the required biomedical data concerning FLI, TSH, FT3 and FT4, liver enzymes and MetS classification. After applying the exclusion criteria (Figure 1) the final study group consisted of 20,289 euthyroid subjects. Figure 1 shows the flow chart for selection of participants.

Figure 1. Flow chart of the study population.



A FLI ≥ 60 was observed in 4,274 participants (21.1% of the study group). Subjects with a FLI ≥ 60 were older, were more likely to be male (62.1%) and to fulfill the criteria for MetS (46.7% vs. 4.2%) (Table 1). ALT, AST, ALP and GGT values were all higher in subjects with a FLI ≥ 60 (all $P < 0.0001$). FT4 levels were lower, whereas FT3 and the FT4/FT3 ratio were higher in subjects with a FLI ≥ 60 ($P < 0.0001$ for each). All these differences between subjects with and without a FLI ≥ 60 remained present after adjustment for age and sex. TSH levels did not differ according to FLI categorization.

Table 1. Clinical and laboratory characteristics in subjects with and without non-alcoholic fatty liver disease estimated by the Fatty Liver Index (FLI) ≥ 60.

	FLI < 60 (N = 16,015)	**FLI ≥ 60 (N = 4,274)**	***P*-value**	***P*-value (adjusted for age and sex)**
Age (years)	42 (33-49)	46 (39-51)	< 0.0001	
Sex (male / female)	6188 (38.6%) / 9827 (61.4%)	2655 (62.1%) / 1619 (37.9%)	< 0.0001	
Metabolic syndrome	668 (4.2%)	1994 (46.7%)	< 0.0001	< 0.0001
- Enlarged waist circumference (yes)	3457 (21.6%)	3379 (79.1%)	< 0.0001	< 0.0001
- Hyperglycemia **≥ 5.6 mmol/L** **≥ 7.0 mmol/L**	 998 (6.2%) 45 (0.3%)	 1073 (25.1%) 88 (2.1%)	 < 0.0001 < 0.0001	 < 0.0001 < 0.0001
- Hypertension (yes)	4677 (29.2%)	2475 (57.9%)	< 0.0001	< 0.0001
- Elevated triglycerides (yes)	899 (5.6%)	1861 (43.5%)	< 0.0001	< 0.0001
- Low HDL cholesterol (yes)	2436 (15.2%)	1864 (43.6%)	< 0.0001	< 0.0001
AST (U/L)	22 (19-26)	25 (21-29)	< 0.0001	< 0.0001
ALT (U/L)	18 (14-24)	28 (20-39)	< 0.0001	< 0.0001
GGT (U/L)	18 (14-24)	33 (24-48)	< 0.0001	< 0.0001
ALP (U/L)	58 (49-69)	67 (57-79)	< 0.0001	< 0.0001
TSH (mU/L)	2.01 (1.49-2.65)	2.04 (1.52-2.66)	0.15	0.05
FT4 (pmol/L)	15.70 (14.60-17.00)	15.40 (14.20-16.70)	< 0.0001	< 0.0001
FT3 (pmol/L)	5.20 (4.90-5.60)	5.30 (5.00-5.70)	< 0.0001	< 0.0001
FT3/FT4 ratio	0.33 (0.31-0.36)	0.35 (0.32-0.38)	< 0.0001	< 0.0001

*Data are given in median with interquartile ranges (IQR) or in numbers with percentages. For continuous variables Mann-Whitney U tests and for binary variables Chi square tests were used. Age- and sex-adjusted p-values were obtained by multivariable linear regression analysis. Fatty Liver Index wax calculated by; FLI = ($e^{0.953*\log e\,(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e\,(\text{GGT}) + 0.053*\text{waist circumference} - 15.745}$) / ($1+e^{0.953*\log e\,(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e\,(\text{GGT}) + 0.053*\text{waist circumference} - 15.745}$) *100.*

*Metabolic syndrome was defined according to NCEP ATPIII criteria. Abbreviations: **ALP**, alkaline phosphatase; **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **FT4**, free thyroxine; **FT3**, free triiodothyronine; **GGT**, gamma-glutamyl transferase; **HDL cholesterol**, High Density Lipoprotein cholesterol; **TSH**, thyroid-stimulating hormone.*

In age- and sex- adjusted analysis a FLI ≥ 60 was independently associated with a higher FT3 (OR 1.34, 95% CI 1.29-1.39, P < 0.0001) and a lower FT4 (OR 0.73, 95% CI 0.70-0.75, P < 0.0001), but not with the TSH level (Table 2; model 1). In alternative analysis, a FLI ≥ 60 was associated with a higher FT3/FT4 ratio (OR 1.44, 95% CI 1.39-1.49, P < 0.0001) (Table 2; model 2). In subsequent analyses now also including the presence of the MetS (Table 3) or the individual MetS components (Table 4), similar independent associations of a FLI ≥ 60 with higher FT3, lower FT4 (model 1) or a higher FT3/FT4 ratio (model 2) were found. In the analyses including MetS (components) the associations of a FLI ≥ 60 with FT4, FT3 (model 1) and the FT3/FT4 ratio (model 2) were modestly attenuated. Besides a strong association of a FLI ≥ 60 with the presence of MetS (OR 17.91, 95% CI 16.17-19.82, P < 0.0001) (Table 3; model 1 and 2), a FLI ≥ 60 was also independently associated with each of the individual MetS components (Table 4; model 1 and 2).

Table 2. Multivariable logistic regression analyses demonstrating independent associations of non-alcoholic fatty liver disease estimated by the Fatty Liver Index (FLI) ≥ 60 with thyroid function parameters.

	Model 1			**Model 2**		
	OR	**CI 95%**	***P*-value**	**OR**	**CI 95%**	***P*-value**
Age (years)	1.03	1.03-1.04	< 0.0001	1.03	1.03-1.04	< 0.0001
Sex (male vs. female)	2.33	2.17-2.51	< 0.0001	2.35	2.19-2.52	< 0.0001
TSH (per SD)	1.02	0.98-1.06	0.31	1.02	0.98-1.06	0.32
FT4 (per SD)	0.73	0.70-0.75	< 0.0001			
FT3 (per SD)	1.34	1.29-1.39	< 0.0001			
Ratio FT3/FT4 (per SD)				1.44	1.39-1.49	< 0.0001

OR: odds ratio. CI 95%: 95% confidence interval. OR's are given per year increase in age, for men vs. women and per 1 SD increase in TSH, FT4, FT3 and the FT3/FT4 ratio and with 95% CI's. Binary logistic regression analysis was used. All models are mutually adjusted for the variables included in the analyses. Fatty Liver Index wax calculated by; FLI = $(e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745})$ */* $(1+e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745})$ **100. Abbreviations:* ***FT4****, free thyroxine;* ***FT3****, free triiodothyronine;* ***TSH****, thyroid-stimulating hormone.*

Table 3. Multivariable logistic regression analyses demonstrating independent associations of non-alcoholic fatty liver disease estimated by the Fatty Liver Index (FLI) ≥ 60 with thyroid function parameters and presence of the metabolic syndrome.

	Model 1			Model 2		
	OR	**CI 95%**	***P*-value**	***OR***	**CI 95%**	***P*-value**
Age (years)	1.02	1.02-1.02	< 0.0001	1.02	1.02-1.03	< 0.0001
Sex (male vs. female)	2.43	2.23-2.64	< 0.0001	2.39	2.20-2.60	< 0.0001
TSH (per SD)	1.01	0.97-1.05	0.59	1.01	0.97-1.06	0.52
Free T4 (per SD)	0.76	0.73-0.80	< 0.0001			
Free T3 (per SD)	1.23	1.18-1.29	< 0.0001			
Ratio T3/T4 (per SD)			< 0.0001	1.34	1.29-1.40	< 0.0001
MetS (yes/no)	17.91	16.17-19.82	< 0.0001	17.86	16.13-19.77	< 0.0001

*vs. women, and per 1 SD in TSH, FT4, FT3 and the FT3/FT4 ratio and with 95% CI's. All models are mutually adjusted for the variables included in the analyses. Fatty Liver Index wax calculated by; FLI = ($e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745}$) / ($1+e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745}$) *100. Metabolic Syndrome was defined according to NCEP ATPIII criteria. Abbreviations:* ***FT4****, free thyroxine;* ***FT3****, free triiodothyronine;* ***MetS****, metabolic syndrome;* ***TSH****, thyroid-stimulating hormone.*

Table 4. Multivariable logistic regression analyses demonstrating independent associations of non-alcoholic fatty liver disease estimated by the Fatty Liver Index (FLI) ≥ 60 with thyroid function parameters and the presence of individual metabolic syndrome components.

	Model 1			Model 2		
	OR	**CI 95%**	***P*-value**	***OR***	**CI 95%**	***P*-value**
Age (years)	1.01	1.00-1.01	0.02	1.01	1.00-1.01	0.01
Sex (male vs. female)	10.52	9.13-12.13	< 0.0001	10.38	9.03-11.92	< 0.0001
TSH (per SD)	1.05	1.00-1.11	0.04	1.05	1.00-1.11	0.04
Free T4 (per SD)	0.81	0.77-0.86	< 0.0001			
Free T3 (per SD)	1.17	1.11-1.23	< 0.0001			
Ratio T3/T4 (per SD)				1.25	1.19-1.32	< 0.0001
Enlarged waist circumference (yes/no)	55.55	47.84-64.51	< 0.0001	55.48	47.77-64.42	< 0.0001
Hyperglycemia (yes/no)	2.42	2.11-2.78	< 0.0001	2.42	2.11-2.78	< 0.0001
Hypertension (yes/no)	1.71	1.54-1.89	< 0.0001	1.70	1.54-1.89	< 0.0001
Elevated triglycerides (yes/no)	10.46	9.18-11.91	< 0.0001	10.47	9.19-11.92	< 0.0001
Low HDL cholesterol (yes/no)	2.34	2.10-2.61	< 0.0001	2.34	2.10-2.61	< 0.0001

*OR: odds ratio. CI 95%: 95% confidence interval. OR's are given per year increase in age, for men vs. women, and per 1 SD in TSH, FT4, FT3 and the FT3/FT4 ratio with 95% CI's. All models are mutually adjusted for the variables included in the analyses. Fatty Liver Index wax calculated by; FLI = ($e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745}$) / ($1+e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745}$) *100. Components of the metabolic syndrome (including hyperglycemia) were defined according to NCEP ATPIII criteria. Abbreviations:* ***FT4,*** *free thyroxine;* ***FT3****, free triiodothyronine;* ***HDL cholesterol****, High Density Lipoprotein cholesterol;* ***TSH****, thyroid-stimulating hormone.*

Table 5. Thyroid function parameters according to enlarged waist circumference.

	Sex-stratified waist circumference		***P*-value**
	Not enlarged N = 9,555 (26.1% male, 73.9% female)	**Enlarged** N = 10,874 (59.1% male, 40.9% female)	
TSH (mU/L)	2.04 (1.51-2.70)	1.99 (1.47-2.62)	< 0.0001
FT4 (pmol/L)	15.70 (14.60-17.00)	15.60 (14.40-16.90)	< 0.0001
FT3 (pmol/L)	5.20 (4.80-5.60)	5.30 (4.90-5.70)	< 0.0001
Ratio FT3/FT4	0.33 (0.31-0.36)	0.34 (0.31-0.37)	< 0.0001

*Data are given in median with interquartile ranges (IQR). Waist circumference was sex stratified. Enlarged waist circumference was defined as ≥ 102 cm in males and as ≥ 88 cm in females. Mann-Whitney U test was used. Abbreviations: **FT4**, free thyroxine; **FT3**, free triiodothyronine; **TSH**, thyroid-stimulating hormone.*

Table 6. Sensitivity analyses demonstrating independent associations of non-alcoholic fatty liver disease estimated by the Fatty Liver Index (FLI) ≥ 90 with thyroid function parameters and presence of the metabolic syndrome.

	Model 1			Model 2			Model 3			Model 4		
	OR	**CI 95%**	***P*-value**	***OR***	**CI 95%**	***P*-value**	***OR***	**CI 95%**	***P*-value**	***OR***	**CI 95%**	***P*-value**
Age (years)	1.02	1.02-1.03	< 0.0001	1.02	1.02-1.03	< 0.0001	1.00	0.99-1.01	0.91	1.00	0.99-1.01	0.97
Sex (male vs. female)	1.65	1.42-1.92	< 0.0001	1.69	1.46-1.95	< 0.0001	1.34	1.14-1.57	< 0.0001	1.35	1.16-1.57	< 0.0001
TSH (per SD)	1.05	0.98-1.13	0.15	1.05	0.98-1.13	0.16	1.05	0.96-1.12	0.33	1.04	0.96-1.12	0.33
Free T4 (per SD)	0.72	0.67-0.78	< 0.0001				0.83	0.77-0.90	< 0.0001			
Free T3 (per SD)	1.38	1.28-1.49	< 0.0001				1.18	1.09-1.29	< 0.0001			
Ratio T3/T4 (per SD)				1.44	1.35-1.54	< 0.0001				1.22	1.14-1.31	< 0.0001
MetS (yes/no)							20.57	17.57-24.38	< 0.0001	20.72	17.58-24.40	< 0.0001

OR: odds ratio. CI 95%: 95% confidence interval. OR's are given per year increase in age, for men vs. women, and per 1 SD in TSH, FT4, FT3 and the FT3/FT4 ratio and with 95% CI's. All models are mutually adjusted for the variables included in the analyses. Fatty Liver Index wax calculated by; FLI = ($e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745}$) / ($1+e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745}$) *100. Metabolic Syndrome was defined according to NCEP ATPIII criteria. Abbreviations: FT4, free thyroxine; FT3, free triiodothyronine; MetS, metabolic syndrome; TSH, thyroid-stimulating hormone.

Table 7. Thyroid function parameters, according to fibrosis, estimated by NAFLD fibrosis score (NFS) and non-alcoholic fatty liver disease, estimated by the Fatty Liver Index (FLI).

	No fibrosis NFS < 0.676 N = 20,171		***P*-value**	**Fibrosis** NFS > 0.676 N = 49		***P*-value**
	No NAFLD FLI < 60 N = 15,931	**NAFLD** FLI ≥ 60 N = 4,240		**No NAFLD** FLI < 60 N = 29	**NAFLD** FLI ≥ 60 N = 20	
TSH (mU/L)	2.01 (1.49-2.65)	2.04 (1.52-2.66)	0.14	2.17 (1.58-2.88)	2.05 (1.69-2.70)	0.95
FT4 (pmol/L)	15.70 (14.60-17.00)	15.40 (14.20-16.70)	< 0.0001	16.10 (14.90-17.30)	14.70 (14.33-16.43)	0.05
FT3 (pmol/L)	5.20 (4.90-5.60)	5.30 (5.00-5.70)	< 0.0001	5.30 (4.95-5.60)	4.95 (4.60-5.45)	0.08
Ratio FT3/FT4	0.33 (0.31-0.36)	0.35 (0.32-0.38)	< 0.0001	0.33 (0.29-0.36)	0.33 (0.31-0.36)	0.78

*Data are given in median with interquartile ranges (IQR). NAFLD fibrosis score was calculated by; NFS = (-1.675 + 0.037 x age (year) + 0.094 x BMI (kg/m^2) + 1.13 x fasting glucose/diabetes (yes = 1, no = 0) + 0.99 x AST/ ALT ratio - 0.013 x platelet count (x10 /L) – 6.6 x albumin (g/L)). Fatty Liver Index wax calculated by; FLI = ($e^{0.953*\log e (triglycerides) + 0.139*BMI +0.718*\log e (GGT) + 0.053*waist\ circumference - 15.745}$) / (1+$e^{0.953*\log e (triglycerides) + 0.139*BMI +0.718*\log e (GGT) + 0.053*waist\ circumference - 15.745}$) *100. Mann-Whitney U test was used. Abbreviations:* ***FT4****, free thyroxine;* ***FT3****, free triiodothyronine;* ***TSH****, thyroid-stimulating hormone.*

Discussion

This large population-based study among strictly euthyroid individuals has demonstrated that NAFLD (defined by FLI ≥ 60) is associated with a higher FT3 and a lower FT4 level. The strongest association with an elevated FLI score was observed for the FT3/FT4 ratio. The relationships of an elevated FLI score with FT3, FT4 and the FT3/FT4 ratio were independent of age, sex, the presence of MetS and its individual components. Furthermore, the FT3/FT4 ratio was higher in subjects with an enlarged waist circumference. Taken together, the present results are in agreement with the possibility that higher FT3 levels within the euthyroid range may contribute to hepatic fat accumulation probably in the context of central obesity.

In the interpretation of the current and previous studies on the relation of thyroid function and NAFLD it should be emphasized that each individual probably has a rather narrow set-point of thyroid function status [5]. This concept underscores the clinical utility of a single dataset of thyroid hormone levels in the evaluation of cardiometabolic disorders. The relationship of FT3 with NAFLD has been studied in a few studies that all used ultrasound for establishing NAFLD [15,17-19]. Except for a study in Chinese subjects, which were also selected to have TSH, FT4 and FT3 levels each within their respective reference range, an association between NAFLD and a higher FT3 has not been previously reported. In this report, TSH and FT4 were not different between subjects with and without NAFLD [19]. In other studies, variable associations of FT4 and TSH within the euthyroid range and NAFLD have been documented with some reports showing an association with lower FT4 [17,18], which is in keeping with the present results, or higher TSH levels [15]. In one of these reports subjects with (mild) thyroid function abnormalities were not excluded [18], whereas in other studies euthyroidism was defined as a TSH and a FT4 level within the reference range without taking the FT3 level into account [13,17]. Thus, it seems plausible that patient selection could at least in part explain the discrepancies between the present and these earlier reports [15,17,18].

Triiodothyronine is commonly believed to be more biologically active as a regulator of metabolic processes than is prohormone, thyroxine [29,30]. Thus from a pathophysiological perspective, the association of NAFLD with a higher FT3 rather than with a lower FT4 should be regarded as the most relevant. Multiple interdependent pathways involved in lipid metabolism are affected by thyroid hormone status. T3 conceivably modifies the accumulation of lipids in liver tissue, and hence is probably involved in the pathogenesis of NAFLD [9]. T3 stimulates lipolysis in adipose tissue, thereby enhancing the availability of free fatty acids, which are subsequently used as substrates for triglyceride synthesis in the liver [8,31,32]. Furthermore, T3 is able to stimulate *de novo* lipogenesis in the liver [33]. In agreement, hepatic lipogenesis is increased in subjects with hyperthyroidism [32]. On

the other hand, a liver-targeted agonist of thyroid hormone receptor β, the subunit that is naturally expressed in hepatocytes, has been shown to diminish hepatic fat accumulation in animal studies [34]. In addition, T3 stimulates beta-oxidation of fatty acids which is anticipated to oppose hepatic lipid accumulation [9,35]. Of note, this process involves hepatic autophagy, which in the long run promotes hepatocyte damage [35], along with excessive production of reactive oxygen species by mitochondria [7,34]. Finally, thyroid hormone action may also directly affect the hepatic secretion of triglyceride-rich lipoproteins. Hyperthyroidism impairs the release of very low density lipoproteins (VLDL) from perfused rat liver [36], whereas subclinical hypothyroidism results in increased hepatic VLDL triglyceride secretion [37]. Taken together, it is plausible that partly opposing effects of T3 on various metabolic pathways involved in hepatic lipid metabolism affects the overall effect of thyroid hormone status on hepatic fat accumulation apparently resulting in an increased prevalence of NAFLD in the context of higher FT3 levels within the euthyroid range. Remarkably among subjects with fibrosis, an elevated FLI was not associated with a higher FT3 or a higher FT3/FT4 ratio, suggesting that thyroid hormone status may predominantly impact on fatty liver as an early stage of NAFLD.

In view of the key role of thyroid hormone status on hepatic fat accumulation and lipoprotein metabolism [5,9,38], much effort has been recently paid to the development of thyromimetics, i.e. thyroid hormone receptor agonists that act by binding to specific thyroid hormone receptor hormone subunits [39,40]. Several of these agents including a liver-targeted agonist of thyroid hormone receptor β, the subunit that is naturally expressed in hepatocytes, has been shown to diminish hepatic fat accumulation in animal studies [34]. However, due to deleterious side effects, none of these drugs has been introduced into clinical practice so far, which underscores the complexity of thyroid hormone physiology [40].

It is increasingly recognized that thyroid hormone levels are associated with effects on body fat, in such a way that (centrally) euthyroid obese individuals have higher circulating FT3 [41-43]. Our current findings with respect to higher FT3 together with lower FT4 and higher TSH levels in centrally obese individuals are consistent with earlier reports [41-43]. Using a Mendelian randomization approach it was documented recently that genetic predisposition for a higher BMI is a determinant of higher FT3 levels. This suggests that adiposity may be causally implicated in the regulation of circulating thyroid hormones [43]. The mechanisms responsible for this effect of adiposity are not yet precisely known, but may involve tissue-specific alterations in iodothyronine deiodinase (DIO) expression in relation to obesity. It has been proposed that type 2 DIO is the major source of circulating T3 in humans [44], while on the other hand common genetic variation in type 1 DIO may predict the plasma FT3/FT4 ratio [45]. Among other tissues, type 1 DIO has been isolated from white adipose tissue, whereas type 2 DIO is expressed in brown adipose tissue [46,47]. Type 1 DIO is also expressed in the liver and is stimulated by leptin [46,48], which

in turn may promote fatty acid oxidation, possibly contributing to hepatocyte damage [49]. The importance of (central) obesity in the pathogenesis of NAFLD is well established, and we expectedly observed a strong association of a FLI ≥ 60 with an enlarged waist circumference. We hypothesize that a higher FT3/FT4 ratio in centrally obese subjects, possibly consequent to altered DIO expression, could to some extent reflect an effect of altered thyroid hormone status on NAFLD under euthyroid conditions. Clearly, the cross-sectional design of the present study hampers to establish a sequence of metabolic changes, and the possible interrelationship of central obesity with DIO activity and the development of NAFLD needs to be prospectively delineated in future.

As recommended by the EASL-EASD-EASO NAFLD guidelines, the FLI algorithm, developed using data of the Dionysos Nutrition & Liver Study in Northern Italy [24], was used for detecting the presence of NAFLD. This score is described in the NAFLD guideline as one of the 3 best-validated steatosis scores so far [23], and has been proven highly accurate in detecting fatty liver (accuracy of 0.84 and specificity of 86%) for an FLI ≥ 60; area under the receiver operating characteristic curve 0.83 [24,50]. The NFS has been developed in a predominantly Caucasian (90%), cohort [25] and is described in the NAFLD guideline as one of the best biomarkers for the detection of fibrosis [23]. This score has been externally validated in ethnically different NAFLD populations, with consistent results [23,27]. Both the FLI and NFS seem to perform best in Caucasians, which is probably related to the ethnical difference in fat distribution [23-25,27]. However, the FLI and NFS scores are not an absolute measure of hepatic fat accumulation and fibrosis. While histological examination of a liver biopsy is still the golden standard for diagnosing NAFLD and fibrosis, liver biopsy also has well-known limitations with respect to invasiveness and sampling variability. As an alternative, imaging techniques are time consuming, expensive and also not feasible in large observational studies. Given these considerations, the recent EASL-EASD-EASO NAFLD guidelines have adopted that serum biomarkers are the preferred diagnostic tool for large scale screening studies [23].

Our study has several strengths. Considering a sample size of approximately 20,000 individuals, this is the largest study to date, which reports on the association of NAFLD with thyroid function parameters in patients with euthyroidism. Additionally, all participants included in the Lifelines Cohort Study have been well characterized, with extensive validated questionnaires and standardized (anthropometric) measurements. Laboratory measurements were performed in fasting serum samples in a single reference laboratory [21]. Furthermore, the Lifelines study population has been validated to be representative of the population of the North of the Netherlands [22]. Several other methodological aspects and limitations of our study need to be considered. First, we excluded subjects with concurrent major diseases including cancer to circumvent bias due to non-thyroidal illness. We also excluded subjects using lipid and glucose lowering medication to obviate

alleged effects on hepatic lipid metabolism and to avoid confounding on the assessment of NAFLD. Consequently, this has resulted in underrepresentation of diabetic and more severe dyslipidemic subjects in our study population. Indeed although 10.2% of subjects had elevated plasma glucose, a fasting plasma glucose ≥ 7.0 mmol/L, as diabetes criterion, was present in only 0.6% of the selected participants compared to 2.5% in the entire Lifelines cohort [22]. Also, subjects of non-white ancestry were excluded in order to select a Western-European population. While this likely limits extrapolation of our findings to other ethnicities, this was done in view of the limited percentage of immigrants in our region (Figure 1), and our choice to use the FLI score and NFS for NAFLD and fibrosis assessment. Second, we performed a cross-sectional study. Therefore, cause-effect relationships cannot be established with certainty. Third, since levels of anti-thyroid peroxidase and anti-thyroglobulin autoantibodies were not measured in the Lifelines Cohort Study, we cannot explore the possible influence of impending thyroid autoimmunity on the relationship of NAFLD with thyroid function. Fourth, since self-reported questionnaires were used for a number of relevant variables, we cannot exclude the possibility that misreporting could have resulted in a limited degree of misclassification. Fifth, the FLI score includes the variables waist circumference, BMI, GGT and TG. Therefore, apparent differences in these variables between subjects with and without an elevated FLI score, as well as the extent to which the corresponding MetS components were associated with an elevated FLI should be interpreted with caution. Finally, in sensitivity analyses increasing the FLI cut-off value from 60 to 90, thereby increasing its specificity at the expense of sensitivity [24], comparable associations of a FLI ≥ 90 with thyroid function parameters were observed, supporting the robustness of our findings.

In conclusion, euthyroid subjects with suspected NAFLD are characterized by a higher FT3, a lower FT4 and in particular a higher FT3/FT4 ratio, probably consequent to central obesity. From a clinical point of view, the present findings suggest potentially adverse consequences of higher T3 exposure within euthyroid range, and would underscore the necessity to evaluate the safety of T4-T3 combination treatment in subjects with overt hypothyroidism on NAFLD development.

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10.

Summary, general discussion and future perspectives

Summary and General Discussion

Low-normal thyroid function, i.e. either a higher TSH or a lower FT4 level within the euthyroid reference range, may contribute to the pathogenesis of atherosclerotic cardiovascular disease (CVD) [1-8]. This thesis focused on the effect of low-normal thyroid function on novel lipid and non-lipid biomarkers which are conceivably involved in the pathogenesis of CVD.

Chapter 1 provides the general introduction and aims of this thesis. First, thyroid hormone secretion and regulation is described. Attention is then focused on the role of thyroid hormones on many metabolic pathways that affect atherosclerotic cardiovascular disease. Furthermore, the relationship of thyroid hormones with components of the metabolic syndrome (MetS) and non-alcoholic fatty liver disease (NAFLD) is described. Biomarkers of CVD which may be associated with low-normal thyroid function, such as apolipoprotein B (apoB)-containing lipoproteins and lipoprotein subfractions, adipokines and tumor necrosis factor alfa (TNF-α), are reviewed. At the end of this section we have described the aim of this thesis: to delineate the relationship of low-normal thyroid function with novel lipid and non-lipid biomarkers which may be involved in the pathogenesis of atherosclerotic CVD, and NAFLD, which shares common pathogenic mechanisms with the process of atherosclerosis.

Chapter 2 is a narrative review that provides detailed information about the relationships of low-normal thyroid function with CVD, chronic kidney disease (CKD), lipids and lipoprotein function, MetS and NAFLD, and the responsible mechanisms for these relationships. This review includes results from previously published systemic reviews and meta-analyses, which are based on clinical and basic research papers. These studies suggest that low-normal thyroid function may be implicated in the pathogeneses of atherosclerotic CVD. Low-normal thyroid function could also play a role in the development of MetS, insulin resistance and chronic kidney disease. However, the relationship of low-normal thyroid function with NAFLD is uncertain.

In **Chapter 3**, we evaluated the relationships of plasma lipids and lipoprotein subfractions with thyroid stimulating hormone (TSH) and free T4 (FT4) in 113 euthyroid subjects and we assessed whether such relationships are modified in the context of Type 2 diabetes mellitus (T2DM). Increased hepatic production of large VLDL is considered to represent an important mechanism responsible for higher plasma triglycerides, as observed in T2DM, obesity and MetS [9-11]. We found that low-normal thyroid function may confer increased plasma triglycerides, large very low density lipoproteins (VLDL) particles and -consistently- a greater VLDL particle size. We also found that these relationships are not to a major extent modified in the context of T2DM. This suggests that interindividual variations

in thyroid function even in the low-normal range may contribute to higher circulating triglycerides consequent to increased large VLDL particles. These results are in line with data showing that the hepatic production of large VLDL particles is elevated in subclinical hypothyroidism [12].

In **Chapter 4**, we showed that low-normal thyroid function may influence the metabolism of triglyceride-rich lipoproteins by affecting apolipoprotein (apo) E. This study included 154 euthyroid subjects with and without T2DM. Plasma triglycerides, non-high density lipoprotein (non-HDL) cholesterol, and apoE levels were each independently and positively associated with TSH after adjustment for age, sex, T2DM and the presence of the APOEε3 allele. After adjustment for triglycerides and non-HDL cholesterol or apoB, the association of apoE with TSH remained present. The presence of T2DM did not influence this association. These data are consistent with the possibility that low-normal thyroid function may impact on the metabolism of triglyceride rich lipoproteins by affecting apoE regulation.

Chapter 5 describes the relationships of plasma pre β-HDL with thyroid function in 154 euthyroid subjects with and without T2DM. This study showed that pre β-HDL formation was positively related to FT4, phospholipid transfer protein (PLTP) activity, total cholesterol and triglycerides in T2DM. This relationship was similarly present when pre β-HDL formation was expressed in plasma apoA-1 concentration or in percentage of plasma apoA-1. In contrast, no such relationship was observed in non-diabetic subjects. This relationship also remained present when taking account of plasma PLTP activity, total cholesterol and triglycerides. These results are consistent with the concept that variation in thyroid function within the euthyroid range may influence the metabolism of pre β-HDL, especially in T2DM. Elevated triglycerides and PLTP activity in T2DM, known to contribute to pre β-HDL formation, could possibly explain why pre β-HDL formation was found to be associated with FT4 in diabetic subjects.

Chapter 6 concerns a large population-based study among strictly euthyroid subjects (n=2206) from the Prevention of Renal and Vascular END-Stage Disease (PREVEND) cohort. The aim of this study was to determine the associations of PON-1 and HDL-associated enzyme with important anti-oxidative properties, with thyroid function parameters. We found that PON-1 activity was positively related to TSH and inversely related to FT4. The inverse relationship of PON-1 activity with free T4 remained present after adjustment for lipids (including HDL cholesterol) and other relevant covariates. The inverse relationship of PON-1 activity with FT4 was not different in subjects with vs. without MetS, nor modified by the presence of its individual components. These results are in agreement with the hypothesis that variations in thyroid function within the euthyroid range may influence PON-1 regulation.

In **Chapter 7** we describe the association of low-normal thyroid function with TNF-α, a pro-inflammatory biomarker. TNF-α has been reported to be involved in the pathogenesis and progression in atherosclerosis [13,14]. This study showed, for the first time, that TNF-α was inversely related to FT4 in 154 euthyroid subjects without Type 2 diabetes. After adjustment for age, sex and thyroid autoantibodies this inverse relationship of TNF-α with free T4 remained present. These data raise the possibility that low-normal thyroid function may contribute to enhanced low-grade chronic inflammation, particularly in non-diabetic subjects. The reasons for the absence of such a relationship in diabetic subjects are unclear at present.

Chapter 8 describes the relationship of the leptin/adiponectin (L/A) ratio with low-normal thyroid function in 153 euthyroid subjects. A higher L/A ratio may reflect adipocyte dysfunction, and is an alleged predictor of CVD [15-17]. This study reveals, to our knowledge for the first time, that the plasma L/A ratio is positively related to a higher TSH level in euthyroid subjects with MetS, but not in subjects without MetS. This relationship remained present when relevant covariates were taken into account. In MetS subjects, the L/A ratio remained positively related with TSH after adjustment for individual MetS components. Our findings support the possibility that low-normal thyroid function could confer increased atherosclerosis susceptibility via an effect on the L/A ratio.
In **Chapter 9** we describe a study performed in the Lifelines Cohort Study in which we determined associations of thyroid hormone parameters with NAFLD among euthyroid subjects. In this study NAFLD was defined by using the fatty liver index (FLI), a score based on serum biomarkers (triglycerides, GGT), waist circumference and body mass index, which has been advocated as an established proxy of NAFLD in epidemiological studies [18,19]. A FLI ≥ 60 was categorized as NAFLD. We found that in age- and sex-adjusted analysis a FLI ≥ 60 was independently associated with a higher FT3 and a lower FT4, but not with TSH. The strongest association with an elevated FLI score was found for the FT3/FT4 ratio. After adjustment for the presence of MetS this association remained statistically significant. Furthermore, FT3 and the FT3/FT4 ratio was higher in subjects with an enlarged waist circumference, consistent with an increased iodothyronine deiodinase expression in adipose tissue. These results are in agreement with the possibility that higher FT3 levels within the euthyroid range may contribute to hepatic fat accumulation probably in the context of central obesity.

General discussion

The concept that low-normal thyroid function is likely to have an adverse impact on atherosclerotic cardio-metabolic disorders is emerging, as evidenced from unfavorable changes in plasma lipoproteins as well as an increased carotid artery intima media

thickness (cIMT) and coronary artery calcification (CAC) [overviewed in chapter 2;2,5-8,20]. The underlying mechanisms that may be responsible for the proposed role of low-normal thyroid function in the pathogenesis of atherosclerotic CVD are complex and not yet completely understood.

Accumulating evidence supports the hypothesis that systemic oxidative stress may contribute to the development of atherosclerosis [21-23]. Low normal-thyroid function may influence oxidative stress: low-normal thyroid function is featured by pro-atherogenic elevations in large triglyceride-rich lipoproteins (chapter 3), which are considered to play a central role in the pathogenesis of low HDL cholesterol. Large VLDL particles, through concerted actions of cholesteryl ester transfer protein (CETP) and lipases, play a pivotal role in the generation of small dense LDL particles, which are prone to oxidative modification [24]. ApoE plays an important role in hepatic VLDL production and impaired VLDL clearance [25,26]. In line, the plasma apoE concentration was found to be elevated in subjects with the metabolic syndrome and in more severely hypertriglyceridemic and hyperglycemic T2DM subjects [27,28]. On the other hand, we have shown that higher plasma apoE relates to low-normal thyroid function, but apoE was not elevated in T2DM. This makes it likely that more profound metabolic dysregulation is required to result in plasma apoE elevations. It is also reported that thyroid function status is directly implicated in affecting apoE regulation, as evidenced in experimental settings, namely *in vitro* and in rat models [29,30]. Collectively, these data make it plausible to postulate that the relationship of apoE with low-normal thyroid function, as documented in this thesis, may reflect a pathogenic mechanism that is involved in the metabolism of VLDL particles, thereby contributing to higher circulating triglyceride levels.

It is widely appreciated that low HDL cholesterol is inversely associated with incident cardiovascular disease [31,32]. However, therapeutic interventions aimed at raising HDL cholesterol do not appreciably improve cardiovascular outcome [33], and it seems likely that HDL functionality is more relevant in this respect than HDL cholesterol levels *per se.* Pre β-HDL particles act as initial acceptors of cell-derived cholesterol via ATP binding cassette transporter A-1 (ABCA1), and hence play an important role in the reverse cholesterol transport pathway, whereby cholesterol is transported from peripheral cells back to the liver for biliary transport and excretion in the feces [34-36]. Although increased pre β-HDL concentrations probably stimulate ABCA1-mediated cholesterol efflux, increased plasma pre β-HDL (formation) levels may paradoxically associate with enhanced atherosclerosis susceptibility [34,37,38]. The responsible mechanisms are not well understood but could reflect impaired HDL maturation resulting in attenuated reverse cholesterol transport. Therefore, it is plausible to postulate that higher (relative) pre β-HDL, as observed in dyslipidemia [39], could represent a biomarker of increased atherosclerosis susceptibility. Studies in rodent models and humans have suggested that the anti-atherogenic effects of the HDL fraction are to a considerable extent attributable to PON-1 activity [40]. A recent

meta-analysis of human studies has demonstrated a linear inverse association between PON-1 activity and CVD risk, which is in part dependent on HDL cholesterol levels [41]. We observed that PON-1 activity was independently and inversely related to FT4. Thus, although the effect is modest, lower thyroid function status within the euthyroid range may confer a higher PON-1 activity. Hence, it seems unlikely that alterations in PON-1 activity are primarily responsible for the increased propensity towards oxidized LDL [24] and the impaired anti-oxidative function of HDL [42] in the context of low-normal thyroid function.

Circulating levels of pro-inflammatory biomarkers may be influenced by thyroid function status. This is in keeping with our findings that TNF-α was inversely related to FT4 in euthyroid subjects. However the regulatory mechanisms whereby low-normal thyroid function relates to higher TNF-α levels are not precisely understood. TNF-α has been reported to be involved in the pathogenesis and progression of atherosclerosis, myocardial ischemia/reperfusion injury and heart failure [13,43]. Higher TNF-α levels in the context of low-normal thyroid function could therefore have functional consequences. TNF-α is associated with endothelial dysfunction in SCH [44]. Furthermore, TNF-α is involved in abnormalities in triglyceride and glucose metabolism in subjects with premature coronary heart disease [45,46]. TNF-α may enhance the production of triglyceride-rich lipoproteins [as showed in chapter 7], an effect, which may in part be mediated by (inhibitory) effects on insulin signaling. Indeed, TNF-α relates positively with plasma triglycerides, as confirmed in this thesis.

Thyroid function status also affects plasma concentrations of leptin and adiponectin, which contribute to the pathogenesis of (obesity-related) atherosclerosis via several interrelated metabolic pathways [47-49]. Leptin contributes to endothelial dysfunction, it stimulates inflammatory reactions, and it may promote hypertrophy and proliferation of vascular smooth muscle cells. In addition, leptin attenuates insulin sensitivity [50,51]. Studies have shown that higher plasma leptin is associated with CVD [15,52]. Adiponectin is known to inhibit the process of atherosclerosis in *in vitro* models, although an association of adiponectin with incident atherosclerotic CVD has been equivocally reported in humans [53-55]. Given that the L/A ratio was found to predict incident CVD independent of established risk factors [15] we used this ratio to demonstrate that low-normal thyroid function relates to a higher L/A ratio.

Besides probable relationships of low-normal thyroid function status with cardiometabolic biomarkers, abnormal thyroid function status has been shown to be associated with NAFLD, a common condition that contributes to increased atherosclerosis susceptibility [56-60]. Indeed, NAFLD is more common in subjects with (subclinical) hypothyroidism [61-64]. Hypothyroidism may also predict its development in the general population [63,64]. However, little is known about the association of variations in thyroid function within the

euthyroid range and NAFLD. In a large population from the north of the Netherlands, i.e. participants from the Lifelines cohort, selected to have TSH, FT4 and FT3 levels each within the euhyroid range, we documented that suspected NAFLD is independently associated with a lower FT4 and a higher FT3 with the strongest association being observed for the FT3/FT4 ratio. Since T3 is believed to be the (most) biologically active thyroid hormone [65-66], we consider the association of NAFLD with FT3 of particular relevance. From a clinical point of view our findings provide a rationale to test the potential adverse effect of T4/T3 combination therapy, sometimes used to treat hypothyroidism, on NAFLD development.

Because each person probably has a rather narrow individual set-point of thyroid function status, it is likely that single measurements of circulating TSH and thyroid hormones provide relevant information regarding the relationship of thyroid function status with cardiovascular and metabolic biomarkers [67-71]. It should be noted that the ranges of TSH, FT4 and FT3 values that were used to define the euthyroid range in the studies making part of this thesis were based on reference intervals from the Laboratory Center of the University Hospital Groningen, the Netherlands (chapter 3-5 and 7-9), or based on those provided by the manufacturer (chapter 6). In this regard it is relevant that thyroid hormone reference intervals vary to some extent between studies, and are still being fine-tuned. Additionally, it should be mentioned that TSH itself could exert direct effects on lipoproteins [72,73], as well as on peripheral T3 metabolism [74], which may require reconsideration of the concept that a “high-normal” TSH level merely reflects the set-point of the pituitary-thyroid axis. From a methodological point, it is also important to note that the studies making part of this thesis are cross-sectional in design. For this reason cause-effect relationships cannot be drawn with certainty. Furthermore, we have previously documented that the relationship of low normal thyroid function (either a higher TSH or a lower FT4 level within the euthyroid reference range) with plasma levels of several biomarkers such as bilirubin, which considered to be a natural anti-oxidant, the antioxidative function of HDL and the process cholesteryl ester transfer (CET), which represents a metabolic intermediate between high plasma triglycerides and low HDL cholesterol, is particularly evident in subjects with hyperglycemia and/or the metabolic syndrome [75-77]. For this reason we decided to perform the studies as described in chapter 3-8 in subjects with and without T2DM or MetS.

Table 1 summarizes the association of variations in thyroid function status within the euthyroid range with lipid and non-lipid biomarkers, and it putative influence on atherosclerotic CVD as documented by our group [5,42,75,76,78,79,80], and in part described in this thesis.

Table 1. The association of thyroid function status with lipid and non-lipid biomarkers, and it putative influence on atherosclerotic CVD as documented by our group and in part described in this thesis.

Biomarkers	Low normal thyroid function	Interaction with subject category +/-	Population
cIMT [ref. 5]	TSH: *ns* FT4: ↓		Non-diabetic subjects
Plasma CET [ref. 76]	TSH: ↑ FT4: ns	T2DM: +	Non-diabetic subjects/ T2DM subjects
Plasma PCSK9 [ref. 78]	TSH: ↑ FT4: ns	Obesity: -	Nonobese subjects/ obese subjects
Plasma Large VLDL [ch. 3 Clin Biochem 2015]	TSH: ns FT4: ↓		Non-diabetic subjects/ T2DM subjects
Plasma apoE [ch. 4 Horm Metab Res 2016]	TSH: ↑ FT4: ns		Non-diabetic subjects/ T2DM subjects
Plasma pre β-HDL [ch. 5 Clin Biochem 2016]	TSH: ns FT4: ↑	T2DM: +	Non-diabetic subjects/ T2DM subjects
HDL antioxidative functionality [ref. 42]	TSH: ns FT4: ↑	Impaired fasting glucose and T2DM: +	Normal fasting glucose impaired fasting glucose/ T2DM
HDL anti- inflammatory function [ref. 79]	TSH: ns FT4: ↓		Non-diabetic subjects/ T2DM subjects
Serum PON-1 activity [ch. 6 Eur J Clin Invest 2018]	TSH: ns FT4: ↓ FT3: ns		General population (T2DM included)
Serum bilirubin [ref. 75]	TSH: ns FT4: ↑	T2DM: +	Non-diabetic subjects/ T2DM subjects
Plasma bilirubin [ref. 80]	TSH: ns FT4: ↑ FT3: ↑	Insulin resistance: +	General population (T2DM excluded)
Plasma TNF- α [ch.7 Horm Metab Res 2017]	TSH: ns FT4: ↓	T2DM: -	Non-diabetic subjects/ T2DM subjects
Plasma L/A-ratio [ch.8 Lipids in Health and Disease 2017]	TSH: ↑ FT4: ns	MetS: +	Non-MetS subjects/ MetS subjects
NAFLD [ch. 9 Metabolism 2017]	TSH: ns FT4: ↓ FT3: ↑		General population (T2DM included)

Abbreviations: apoE: apolipoprotein E; cIMT: carotid artery intima media thickness; CET: cholesteryl ester transfer; FT4: free thyroxine; FT3: free triiodothyronine; HDL: high density lipoprotein; L/A: leptin/adiponectin; MetS: metabolic syndrome; NAFLD: non-alcoholic fatty liver disease; PON-1: paraoxonase-1; T2DM: Type 2 diabetes mellitus; TNF- α: tumor necrosis factor alfa; TSH: thyroid-stimulating hormone; VLDL: very low density lipoprotein. ns: no significant effect; positive (↑); inverse (↓) association.
interaction: + positive; - negative.

Conclusions and future perspectives

The studies described in this thesis mostly point to putative adverse effects of low-normal thyroid function on lipid and non-lipid biomarkers which relate to enhanced susceptibility to atherosclerotic CVD. On the other hand, higher FT4 levels, even in the euthyroid range were reported very recently to associate with increased coronary artery calcification (CAC) and incident atherosclerotic CVD [81]. These findings are clearly at odds with earlier reports demonstrating that low-normal thyroid function is associated with enlarged cIMT and increased CAC [5-8], as well as with a lack of effect of variations in the TSH level within the euthyroid reference range and incident coronary heart disease [3].

Furthermore, it is noteworthy that variations in thyroid function within the reference range impact on many pathological states [2,82,83]. In the context of the thyroid studies collaboration, it has been recently demonstrated that subclinical hypothyroidism is associated with increased risk of (fatal) stroke particularly in younger people [84]. On the other hand, a high-normal FT4 may associate with sudden cardiac death [85] and predict increased incidence of atrial fibrillation. In addition, low-normal thyroid function may associate with incident T2DM [86], in agreement with earlier findings suggesting that several MetS components relate to low-normal thyroid function [87-92]. Furthermore, it has been reported very recently that high-normal FT4 levels are likely to be associated with the development of solid cancer [93]. Still the pathogenic mechanisms responsible for such an association are not immediately apparent. In the near future, an individual participant meta-analysis with regard to the association of thyroid function status and cancer incidence will be carried out within the framework of the Thyroid Studies Collaboration.

As a result of these partly opposing effects of thyroid function status on a number of morbidities, it is difficult to predict the influence of low-normal thyroid function on life expectancy as an integrative approximation of health status. For this reason we have determined whether higher TSH, lower FT4 and FT3 and positive anti-thyroid peroxidase (anti-TPO) autoantibody status would influence life expectancy among euthyroid participants from the PREVEND cohort. This analysis did not reveal an effect of either higher TSH, lower FT4, lower FT3 and anti-TPO autoantibody status on life expectancy [94]. Using a different statistical approach, low normal thyroid function was published to be associated with a longer life expectancy in the Rotterdam study [95]. As yet the reasons for these apparent discrepancies are unclear.

The issue of whether variation in thyroid function status may impact on clinically important morbidities is currently widely studied with contrasting results being published during the

past few years. It is expected that more insight will be obtained when additional meta-analyses become available. At present, measurement of thyroid function, still poses challenges in interpretation and applicability for the individual patient. It remains unclear if measurement of thyroid function leads to a better therapeutic regimen to reduce cardiovascular risk. From a clinical perspective, it is relevant to identify those subject categories that might benefit from thyroid hormone supplementation not only in the case of SCH [96] but also in the context of low-normal thyroid function. In addition, it remains important to identify new biomarkers involved in the pathogenesis of atherosclerosis in patients with low-normal thyroid function.

Conclusion

Taken together our cross-sectional studies provide evidence that low normal thyroid-function is associated with pro-atherogenic abnormalities in plasma (apo)lipoproteins and inflammation biomarkers. Besides probable relationships of low-normal thyroid function status with cardiometabolic biomarkers, a high-normal FT3 level could also be implicated in the development of NAFLD.

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11.

Nederlandse samenvatting

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Een laag-normale schildklierfunctie, gedefinieerd als een hogere TSH waarde en/of een lagere FT4 waarde binnen het euthyreote referentiegebied, kan een rol spelen bij het optreden van atherosclerotische cardiovasculaire ziekten (CVZ). De onderliggende mechanismen, die mogelijk verantwoordelijk zijn voor de pathogenese van atherosclerotische CVZ bij laag-normale schildklierwaarden, zijn complex en nog niet volledig begrepen.
Dit proefschrift richt zich op het effect van laag-normale schildklierwaarden op (nieuwe) cardiovasculaire biomarkers (lipiden en niet-lipiden) die betrokken kunnen zijn bij de pathogenese van atherosclerotische CVZ.

Hoofdstuk 1 geeft achtergrondinformatie over schildklierfunctie en beschrijft de doelstellingen van dit proefschrift. De rol van schildklierhormonen op verschillende metabole routes, die van invloed kunnen zijn op atherosclerotische cardiovasculaire aandoeningen, worden belicht. Verder wordt de relatie van schildklierhormonen met componenten van het metabool syndroom (MetS) en leververvetting (non alcoholic fatty liver disease (NAFLD)) toegelicht. Verschillende cardiovasculaire biomarkers, als apolipoproteïne B (apoB)-bevattende lipoproteïnen, lipoproteïne subfracties, adipokines en tumornecrosefactor-alfa (TNF-α), worden beschreven.

Hoofdstuk 2 behelst een review over de relatie tussen laag-normale schildklierwaarden met CVZ, chronische nierziekten (CKD), lipiden en lipoproteïnen, MetS en NAFLD. De achterliggende mechanismen voor deze associaties worden belicht. Dit review omvat de resultaten van eerder gepubliceerde systemische reviews en meta-analyses, gebaseerd op klinisch en fundamenteel onderzoek. Deze studies suggereren dat laag-normale schildklierwaarden mogelijk betrokken zijn bij de pathogenese van atherosclerotische CVZ. Laag-normale schildklierwaarden zouden ook een rol kunnen spelen in de ontwikkeling van MetS, insulineresistentie en chronische nierziekte. De relatie van laag-normale schildklierwaarden met NAFLD is echter onzeker.

Hoofdstuk 3 beschrijft de relatie tussen plasma lipiden en lipoproteïne subfracties met schildklier stimulerend hormoon (TSH) en vrij T4 (FT4) bij 113 euthyreote deelnemers. We hebben tevens onderzocht of deze mogelijke relatie verschilt indien er sprake is van Type 2 diabetes mellitus (T2DM). Verhoogde hepatische productie van grote “very low density lipoproteine” (VLDL) deeltjes zorgt voor toename van de plasmatriglyceridenconcentratie. Aandoeningen die gepaard gaan met een hogere plasmatriglyceridenconcentratie zijn onder andere T2DM, obesitas en MetS. We vonden dat een laag-normale schildklierfunctie (met name hoog-normaal TSH) gerelateerd is aan een verhoogde plasmatriglyceridenconcentratie, toename van grote VLDL-deeltjes en een toename

in grootte van VLDL-deeltjes. Daarnaast hebben we vastgesteld dat deze associaties niet verschillen tussen personen met en zonder T2DM. Dit suggereert dat variaties in schildklierfunctie, zelfs in het laag-normale referentiegebied, kunnen bijdragen aan hogere circulerende triglyceriden spiegels door toename van grote VLDL-deeltjes. Deze resultaten komen overeen met onderzoeken die aantonen dat de hepatische productie van grote VLDL-deeltjes verhoogd is bij subklinische hypothyreoïdie.

In **Hoofdstuk 4** laten we zien dat laag-normale schildklierfunctie het metabolisme van triglyceride-rijke lipoproteïnen via apolipoproteïne E (apoE) kan beïnvloeden. Deze studie omvat 154 euthyreote-deelnemers met en zonder T2DM. Plasmatriglyceriden, non-"high density lipoproteïine" (HDL) cholesterol en apoE niveaus waren elk onafhankelijk en positief geassocieerd met TSH, na correctie voor leeftijd, geslacht, T2DM en de aanwezigheid van het APOEε3 allel. Na correctie voor triglyceriden en non-HDL-cholesterol of apoB bleef de associatie van apoE met TSH bestaan. De aanwezigheid van T2DM had geen invloed op deze associatie. Deze resultaten laten zien dat een laag-normale schildklierfunctie invloed kan hebben op het metabolisme van triglyceride-rijke lipoproteïnen via regulering van apoE.

Hoofdstuk 5 beschrijft de relatie tussen plasma pre β-HDL en de schildklierfunctie bij 154 euthyreote deelnemers met en zonder T2DM. De resultaten laten een positieve relatie zien tussen plasma pre β-HDL en FT4, phospholipiden transfer proteïne (PLTP) activiteit, totaal cholesterol en triglyceriden bij personen met T2DM. Deze relatie was hetzelfde wanneer pre β-HDL werd uitgedrukt als de apoA-1-concentratie of in percentage apoA-1 in plasma. Echter, deze relatie werd niet gevonden bij deelnemers zonder diabetes mellitus. Deze relatie bleef aanwezig wanneer er rekening werd gehouden met plasma PLTP activiteit, totaal cholesterol en triglyceriden. Deze resultaten suggereren dat variatie in de schildklierfunctie binnen het euthyreote referentiegebied het metabolisme van pre β-HDL in T2DM kan beïnvloeden.

In **Hoofdstuk 6** beschrijven wij een grote populatie-gebaseerde studie onder euthyreote deelnemers (n=2206) van het Prevent of Renal en Vascular END-Stage Disease (PREVEND) cohort onderzoek. Het PREVEND onderzoek is een onderzoek dat opgezet is om de rol van verhoogde albumine excretie in de urine in de ontwikkeling van nierziekten en CVZ te onderzoeken. Het doel van deze studie was om associaties tussen serum paraoxonase-1 (PON-1) en schildklierfunctieparameters, lipiden en apoA-1 te bepalen. PON-1 is een enzym dat in belangrijke mate bijdraagt aan de anti-oxidatieve eigenschappen van HDL. We vonden dat PON-1 activiteit positief gecorreleerd is met TSH en omgekeerd gecorreleerd met FT4. De omgekeerde correlatie tussen PON-1-activiteit en vrij T4 bleef aanwezig na correctie voor lipiden en andere relevante covariabelen. Deze omgekeerde correlatie

tussen PON-1-activiteit en FT4 was hetzelfde bij deelnemers met en zonder MetS, tevens werd deze relatie niet beïnvloed door aanwezigheid van de afzonderlijke componenten van MetS. Deze resultaten suggereren dat variaties in de schildklierfunctie binnen het euthyreote referentiegebied de regulatie van PON-1 kunnen beïnvloeden.

Hoofdstuk 7 beschrijft de associatie tussen een laag-normale schildklierfunctie en TNF-α, een pro-inflammatoire biomarker. Het is gevonden dat TNF-α betrokken is bij de pathogenese en progressie van atherosclerose. Onze studie laat voor de eerste keer zien dat TNF-α omgekeerd gecorreleerd is met FT4 bij 154 euthyreote deelnemers met en zonder T2DM. Na correctie voor leeftijd, geslacht en schildklier auto-antistoffen bleef deze omgekeerde correlatie tussen TNF-α met vrij T4 aanwezig. Deze resultaten laten zien dat een laag-normale schildklierfunctie (met name een laag vrij T4) kan bijdragen aan een toename van chronische laaggradige inflammatie.

Hoofdstuk 8 beschrijft de relatie tussen de leptine/adiponectine (L/A)-ratio en een laag-normale schildklierfunctie. Dit werd onderzocht bij 153 euthyreote personen. Een hogere L/A-ratio kan wijzen op adipocytdisfunctie en is veronderstelde voorspeller voor CVZ. Deze studie laat voor het eerst zien dat de plasma-L/A-ratio positief is geassocieerd met een hogere TSH waarde bij euthyreote personen met MetS, maar niet bij deelnemers zonder MetS. Deze associatie bleef aanwezig ondanks het in beschouwing nemen van verschillende covariabelen. Na correctie voor de verschillende componenten van MetS bleef de L/A-ratio positief geassocieerd met TSH bij de deelnemers met MetS. Onze bevindingen ondersteunen de mogelijkheid dat een laag-normale schildklierfunctie het risico op atherosclerose kan verhogen via positieve beïnvloeding van de L/A-ratio.
In **hoofdstuk 9** beschrijven we een studie onder 20,289 euthyreote deelnemers van de de Lifelines Cohort Study. Het doel van deze studie was om associaties tussen schildklierhormoonparameters en NAFLD onder euthyreote deelnemers te onderzoeken. In deze studie is de “fatty liver index” (FLI) gebruikt om NAFLD te classificeren. De FLI is gebaseerd op serum triglyceriden, serum GGT, de middelomtrek en body mass index. Een FLI-score ≥ 60 wordt beschouwd als NAFLD equivalent. We vonden dat, na aanpassing voor leeftijd en geslacht, een FLI-score ≥ 60 onafhankelijk geassocieerd was met een hogere FT3 en een lagere FT4, maar niet met TSH. De associatie tussen een verhoogde FLI-score en de FT3/FT4-ratio was het sterkst. Na correctie voor MetS bleef de associatie statistisch significant. De FT3/FT4-ratio was hoger bij personen met een vergrote tailleomtrek. Wij hebben geconcludeerd dat hogere FT3-waarden binnen het euthyreote referentiegebied kunnen bijdragen aan het optreden van leververvetting, waarschijnlijk in het kader van centrale obesitas.

Conclusies en toekomstperspectieven

De meeste studies die beschreven zijn in dit proefschrift, wijzen op een vermoedelijk nadelig effect van een laag-normale schildklierfunctie op cardiovasculaire biomarkers (lipiden en niet-lipiden) en de ontwikkeling van atherosclerotische CVZ. Recent werden hogere FT4-waarden binnen het euthyreote referentiegebied in verband gebracht met toename van coronairsclerose en atherosclerotische CVZ. Deze bevindingen komen niet overeen met verschillende eerdere studies die aantonen dat een laag-normale schildklierfunctie geassocieerd is met een toename van de intima-media dikte van de carotis arteriën en een toename van coronaire calcificaties. Ook zijn er studies die geen effect laten zien van een hogere of lagere TSH-waarde binnen het euthyreote referentiegebied op de incidentie op coronaire hartziekten.

Het is opmerkelijk dat een hoog-normale of laag-normale schildklierfunctie van invloed kan zijn op zoveel verschillende aandoeningen. De Thyroid Studies Collaboration heeft onlangs aangetoond dat subklinische hypothyreoïdie geassocieerd is met een verhoogd risico op een (fatale) cerebrovasculair accident (CVA), met name bij jongeren. Tevens werd gevonden dat een hoog-normale FT4-waarde geassocieerd is met plotse hartdood en een verhoogde incidentie op atriumfibrilleren. Verder werd beschreven dat een laag-normale schildklierfunctie geassocieerd kan zijn met een verhoogd risico op T2DM, wat in overeenstemming is met eerder onderzoek, waarin gesuggereerd wordt dat de verschillende componenten van MetS geassocieerd zijn met een laag-normale schildklierfunctie. Zeer recent is beschreven dat hoog-normale FT4-waarden mogelijk geassocieerd zijn met de ontwikkeling van solide tumoren, echter de mechanismen die hiertoe leiden zijn nagenoeg niet bekend. Toekomstige meta-analyses verricht door de Thyroid Studies Collaboration zouden meer inzicht kunnen geven in de associatie tussen de variatie in schildklierfunctie en de incidentie van kanker.

Vanwege deze deels tegenovergestelde resultaten van schildklierfunctiestatus op een aantal ziekten, is het moeilijk om de invloed van een laag-normale schildklierfunctie op de levensverwachting te voorspellen. Om deze reden hebben wij onderzocht of een hogere TSH, een lagere FT4-waarde, een lagere FT3-waarden, en de aan- of afwezigheid van anti-thyroid peroxidase (anti-TPO) de levensverwachting van euthyreote deelnemers van de PREVEND studie beïnvloed. Deze analyse toonde aan dat er geen effect was van een hogere TSH, een lagere FT4-waarde, een lagere FT3-waarden, en de aan- of afwezigheid van anti-TPO op de levensverwachting. In de Rotterdam Study werd, met een andere statistische benadering, wel een associatie aan tussen een laag-normale schildklierfunctie en een langere levensverwachting gevonden. De oorzaak van deze schijnbare discrepantie is onduidelijk.

De vraag of een laag-normale schildklierfunctie van invloed kan zijn op klinisch belangrijke (cardiovasculaire) ziekten, wordt momenteel uitgebreid bestudeerd. De resultaten die de afgelopen jaren zijn gepubliceerd zijn tegenstrijdig. Meta-analyses zouden meer inzicht kunnen geven. De interpretatie van de schildklierfunctie bij de individuele patiënt en de toepasbaarheid in de klinische praktijk blijft een uitdaging. Het blijft onduidelijk of het meten van de schildklierfunctie leidt tot een beter behandeling met als resultaat het verminderen van het cardiovasculaire risico. Het is klinisch relevant om de subgroepen te identificeren, die baat kunnen hebben bij schildklierhormoon suppletie, niet alleen bij subklinische hypothyreoïdie, maar ook in de context van een laag-normale schildklierfunctie. Daarnaast blijft het belangrijk om nieuwe biomarkers te identificeren die betrokken zijn bij de pathogenese van atherosclerose bij patiënten met een laag-normale schildklierfunctie.

Samenvattend tonen de cross-sectionele studies die beschreven zijn in dit proefschrift aan, dat een laag-normale schildklierfunctie geassocieerd is met pro-atherogene veranderingen in plasma (apo) lipoproteïnen en inflammatoire biomarkers. Naast het feit dat een laag-normale schildklierfunctie geassocieerd kan zijn met cardiovasculaire biomarkers (lipiden en niet-lipiden), zou een hoog-normale FT3-waarde betrokken kunnen zijn bij de ontwikkeling van NAFLD.

Dankwoord

Dankwoord

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Biografie /biography

Biografie

Lynnda van Tienhoven-Wind werd op 24 december 1979 geboren in Groningen. Haar middelbare school doorliep zij aan het Ommelander College in Appingedam, waar zij in 1998 haar Vwo-diploma behaalde. Omdat zij uitgeloot werd voor de opleiding Geneeskunde startte zij de opleiding Medische Biologie. Zij rondde deze opleiding af in 2003 en startte alsnog met de opleiding Geneeskunde aan de Rijksuniversiteit Groningen. Na haar afstuderen in 2009 ging zij als arts-assistent niet-in-opleiding aan het werk bij de afdeling Interne Geneeskunde in het Martini Ziekenhuis te Groningen. In 2011 startte Lynnda de opleiding tot internist. Tijdens haar stage endocrinologie in het vierde jaar van haar opleiding raakte zij in contact met Dr. R.P.F. Dullaart. Samen met Dr. R.P.F. Dullaart schreef zij meerdere artikelen op het gebied van cardiovasculaire biomarkers en laag-normale schildklierfunctie. Deze samenwerking resulteerde in de totstandkoming van dit proefschrift.

Lynnda van Tienhoven-Wind was born in Groningen, the Netherlands, on December 24th of 1979. In 1998 she graduated from high school in Appingedam. After missing the Numeris Fixus for the study Medicine, she started her study Medical Biology, from which she graduated in 2003. At that point she was offered the opportunity to study Medicine at the University of Groningen. After her graduation in 2009 she started a job as a resident Internal Medicine. In 2011 Lynnda started her education to become an internist. During the 4th year of her internship endocrinology she came into contact with met Dr. R.P.F. Dullaart. Together with met Dr. R.P.F. Dullaart she wrote multiple articles in the field of cardiovascular biomarkers and low-normal thyroid function. This cooperation resulted in the realization of this thesis.

List of publications

List of publications

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